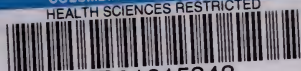


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The
Journal of Infectious Diseases

Published by the John McCormick Institute for Infectious Diseases

EDITED BY
LUDVIG HEKTOEN AND EDWIN O. JORDAN

IN CONJUNCTION WITH
FRANK BILLINGS F. G. NOVY
H. GIDEON WELLS KARL F. MEYER

Volume 31
1922

Chicago, 1922

Composed and Printed by
American Medical Association Press
Chicago, Illinois, U. S. A.

EXPERIMENTAL PRODUCTION OF BOVINE MASTITIS WITH STREPTOCOCCI AND OTHER BACTERIA

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Certain aspects of the etiology of bovine mastitis have been studied but the majority of workers have dealt with organisms from milk from infected udders. The bacteria isolated have fallen, for the most part, into two groups, hemolytic and nonhemolytic streptococci. A smaller percentage is due to the staphylococci, *B. pyogenes* and members of the colon-aerogenes group. In the literature are few references to the experimental production of mastitis. This is due, in all probability, to the fact that the experiment is an expensive procedure because one cannot carry it on without a possibility of damaging the value of the animal.

Franck¹ produced mastitis by introducing infected fluids into the teat canal then injecting the milk from the diseased udder into that of a normal animal. Guillebeau and Hess² state that they have injected subcutaneously into goats an organism which they call *B. coli*, isolated from a case of mastitis, the result being localization of the organism in the mammary gland of the goat followed by a severe inflammation of the udder. Kitt³ states that he has produced mastitis in cattle by immersing the end of the teat in a fluid culture of colon bacilli. Jensen⁴ and Fauss⁵ produced an acute mastitis with *B. coli* and other bacteria. Bang⁶ and Wall⁷ caused mastitis in cattle with streptococci, staphylococci, colon bacilli and *Bacillus pyogenes*. Bang introduced the various organisms into the udder by means of a glass rod dipped in the culture. Meyer⁸ infected 2 animals with streptococci. He states that the inflammation is caused by the metabolic products of the organism and not by the streptococcus itself. Harrison and Cumming⁹ smeared the ends of the teats of the cows with *B. prodigiosus*, *B. fluorescens liquefaciens* and an organism they called *Bact. exiguum* which is a chromogenic bacterium found occasionally in water and milk. They found comparatively small numbers of the organisms in the fore milk for the first 2 or 3 milkings. They did not recognize any inflammation of the udder. Gminder¹⁰ produced mastitis in goats by injecting streptococci

Received for publication March 10, 1922.

¹ Deutsch. Ztschr. f. Tiermed., 1876, 2, p. 456.

² Kolle and Von Wassermann's Handbuch der path. Mikroorganismen, 1913, 6, p. 96.

³ Lehrbuch der pathologischen Anatomie der Haustiere, 1900, 1, p. 200.

⁴ Lubarsch and Ostertag's Ergebnisse der allgem. Pathologie, 1897, 4, p. 830.

⁵ Berner Diss., 1908.

⁶ Tidskrift för Veterinärer, 1888, 18, p. 226.

⁷ Die Enterentzündungen der Kuh, 1908.

⁸ Arch. Wiss. u. Prakt. Tierheilk., 1910, 36, p. 583.

⁹ Jour. Applied Microscopy, 1902, 5, p. 2086.

isolated from chronic cases in cattle. Savage¹¹ failed to produce mastitis in goats with streptococci from tonsillitis and other diseases of man, with the exception of 2 cases in which he observed a slight inflammation of the udder. He introduced the material into the teat canal with a platinum needle. In connection with an outbreak of septic sore throat, which was traced to an infected milk supply, Davis and Capps¹² smeared the uninjured surface of the teats of a cow 3 successive days with cultures of hemolytic streptococci isolated from cases of tonsillitis. Two strains were used in the experiment. The results were negative. Later they made a similar experiment, after an abrasion had been made near the meatus of the teat canal, with positive results. They also introduced well up into the teat canal, by means of a catheter, 8 cc of a 24-hour milk culture of a hemolytic streptococcus, producing a typical mastitis. Mathers¹³ produced mastitis in cattle with virulent hemolytic streptococci from bovine mastitis and from a fatal case of streptococcic peritonitis in man. In neither case did the quarter recover. He produced acute inflammation of the udder with a nonvirulent, nonhemolytic *Streptococcus lacticus* from which infection the quarter returned to normal in a short time. A condition similar to that produced by *Streptococcus lacticus* was brought about by a culture of a nonvirulent hemolytic streptococcus such as is found in normal milk. From 1 to 5 cc of the living culture was injected directly into the teat canal in the different experiments.

Most of the earlier workers with the exception of those mentioned used 10 and 12 cc of a broth culture of the different organisms and injected the material into the canal.

My work was undertaken to ascertain the ability of certain bacteria to produce mastitis in cattle. These organisms, with the exception of *Bact. pyocyaneum* and *Pasteurella bovis septica*, were isolated from cases of mastitis and from diseased genital organs of cattle. I was particularly interested in the question of immunity to these diseases of the udder, whether there is immunity after recovery from this condition, and whether immunity can be established by injecting the organism into the tissues of the body other than the udder.

It is common knowledge that cows put on test for production of milk and butterfat are susceptible to attacks of mastitis, especially when large amounts of food with a high protein content are fed to the animals, but the source of the infection is still unsatisfactorily explained. While doing some work with calves in a large purebred herd, I became keenly interested in this condition, but the value of the animal would not permit any experimental studies. Bacteriologic examinations from four cases showed that the milk contained a nonhemolytic streptococcus and large numbers of leukocytes. As this condition had occurred before in the herd, every precaution was taken by the tester and herdsman to eliminate any external source of infection. The tester disinfected his

¹⁰ Centrallbl. f. Bakteriöl., 1912, 63, p. 152.

¹¹ Rep. of Medical Officer, Local Gov't. Board of London, 1906, '07, '08, '09.

¹² Jour. Infect. Dis., 1914, 15, p. 135.

¹³ Ibid., 1916, 19, p. 222.

hands before milking each animal, and the teats were immersed in an antiseptic before and after each milking. Other sanitary conditions were unusually good, necessarily so because the milk was being supplied for a city.

Thirteen animals ranging in age from $2\frac{1}{2}$ to 11 years, and with apparently normal udders, were used for this work. A bacteriologic examination was made of the milk from the 4 quarters of each udder before the injection of the different organisms. The milk was collected in 4-ounce sterile containers after the ends of the teats had been disinfected with 2% phenol solution and the fore milk discarded. The medium used in the work was a veal infusion agar to which was added sterile horse serum or corpuscles. One-tenth of a cc of milk from the samples was cultivated after shaking thoroughly. Smears also were made from the samples with milk and stained with methylene blue. In 4 of the cows no bacteria were recovered from the milk. In the other cases a few colonies of micrococci producing different pigments, chiefly of the albus and aureus varieties, were found. No streptococci were cultivated from the milk. Occasionally a cell or organism was observed in the smears, but this was not considered as signifying that inflammation existed in the udder.

With the exception of the specific cases mentioned, all cultures used for injection were 24-hour broth cultures. The material was injected into the milk duct by means of a small teat cannula fitted to a 10 cc record syringe. The tip of the cannula was inserted just past the sphincter muscle at the end of the teat and injected at this place. The cultures were injected immediately after the animal had been milked, which was about 4 p. m. The animals were milked by one man with occasional assistance from me. The first personal observation was made about 22 hours after the injection, when the samples were collected, and these were examined and new samples collected once every day thereafter. The report of the condition of the udder and of the animal at the first milking after the injection, which was at 4 a. m., was given to me by the herdsman.

Samples of mastitis milk containing nonhemolytic streptococci were injected into one quarter of the udder of each of 7 animals. Three samples of milk, designated as A, B and C, were obtained from 3 different herds in which mastitis existed in varying degrees. One heifer received 5 cc of sample A 15 minutes before the animal was milked dry. Three older cows, one not in milk (R34), received injections of 5 cc of this same sample A. The aged cow (R34) mentioned in the foregoing, which was dry, received in another quarter 1 cc of sample B. A heifer also received 0.5 cc of this sample.

Sample A proved to be very virulent. Two of the animals suffered from a general reaction and rise in temperature which was followed by a severe diarrhea for 2 days. The third animal did not show the general reaction, but the condition of udder and milk was similar to that of the other 2. The quarter became greatly enlarged, hot and painful, and the material obtained from it in each case was composed of yellowish, stringy pus. The quarters finally atrophied, but they still gave a little pus from which the nonhemolytic streptococcus could be isolated for 4 months, at which time the animals were slaughtered.

The dry cow did not show signs of a reaction from the infection of sample A. The organism was isolated once from the quarter 24 hours after the injection and a few leukocytes were present in the smears, but these appeared greatly disintegrated and were probably the cells that were in the sample that was injected.

The heifer that received 0.5 c.c. of sample B gave a similar general and local reaction to the injection as did the cows mentioned in the foregoing. The quarter atrophied and leukocytes and streptococci were isolated until the time of slaughter. R 34 showed a few similar cells in the secretion obtained from the udder, but the quarter showed no evidence of inflammation. This condition disappeared in a few days.

A quarter of R 51 was injected with 1 c.c. of a 24-hour broth culture of a nonhemolytic streptococcus isolated from the milk sample C. This produced a severe general reaction. The quarter enlarged and became hot and painful. The milk changed to a thick, stringy, yellowish pus. Later the quarters atrophied, and the condition continued the same for a month when the animal was slaughtered. There were large numbers of cells and streptococci present at this time.

Three older animals were also injected with 1 c.c. of a nonhemolytic streptococcus from milk sample B. The cultures of this strain did not prove to be very virulent to these animals. In one case there was no reaction after the injection. The other 2 cases revealed a little pus in the milk, but the udders remained normal. The leukocytes cleared up 8 days after injection, and the streptococcus was not found after the sixth day.

Samples of milk containing hemolytic streptococci were obtained from 3 herds. These are designated as samples 1, 2 and 3. Two aged cows giving milk and R 34 not in milk (the same animal that was used before) were injected with 3 c.c. of sample 1. One animal received 1 c.c. of sample 2 and one animal received 1 c.c. of sample 3. R 34 showed no evidence of infection after the injection of sample 1. The hemolytic streptococcus was recovered from the 24 and 48 hour samples. No polymorphonuclear leukocytes were found in the smears. One animal had a general reaction and the quarter became enlarged, hot and tender. All that could be obtained from the quarter was a small amount of pus that contained large numbers of the organism. This resulted in the atrophy of the quarter, but the milk returned to normal 25 days after injection. The third animal that received the injection from sample 1 was nearly dry, and the quarter apparently was not enlarged or inflamed. The milk showed a few cells and also the organism for 3 days. The quarter and milk were normal on the fifth day after the injection.

Four cultures of a hemolytic streptococcus isolated from samples 1, 2, 3 and from a fourth sample were used on several animals. One quarter of the udder of each of 3 animals, one aged cow and 2 heifers, received 1 c.c. of a 24-hour broth culture of sample 1. The aged cow apparently suffered no ill effects from

the injection. The quarters of the heifers showed a slight swelling and a few flocculi of pus in the milk. One animal was slaughtered 10 days after the injection, and she still showed a few cells and a large number of streptococci at that time. The cells and bacteria in the second heifer disappeared 6 days after she received the injection.

Injections of cultures from milk sample 2 were made on 2 cows and 2 heifers, one of which had progressed about 6 months in her first pregnancy and had never given milk. This heifer and one of the cows failed to show a reaction after receiving the material, but the culture was isolated from this cow for 3 milkings afterward. One heifer and one cow showed a slight inflammation of the udders and a few flocculi of pus in the milk. In these 2 animals the condition remained the same until they were slaughtered.

A culture from sample 3 was injected into one heifer. This produced a slight swelling of the udder and a little pus in the milk. The condition continued for one month at which time the animal was slaughtered.

Three cc of a 24-hour broth culture from sample 4 was injected into one animal that had suffered from mastitis due to a natural infection, the cause of which was not determined. This produced a slight inflammation of the udder and a few leukocytes in the milk, but both conditions cleared up in 3 days. The injection was repeated 6 days later with negative results.

A quarter from the udder of each of eight different animals was injected with *Streptococcus viridans*. Three different cultures of this organism which had been isolated from diseased ovaries and uteri of cattle were used.

Each of 3 aged animals received 6 cc of one culture. One of the cows was not in milk. Three 3-year old heifers were given 2 cc of the second culture and one 5-year old cow and another 3-year old heifer received 2 cc of the third culture.

The results of these injections were quite similar in all cases. As a rule, the inflammation was at its height in 24 hours. Three animals showed no clinical evidence of any inflammation. In 2 other cases, the samples showed no bacteria or leukocytes. In the remaining animals there was a slight but variable manifestation of an inflammation of the quarter. The quarters were rather firm to the touch and somewhat enlarged. Usually the observable inflammation subsided in 2 days, and the samples of milk showed no cells or streptococci 7 days later, with the exception of the milk of the dry cow. The small amount of milk in her udder thickened until it was practically nothing but strings of pus. It was 22 days before the cells and bacteria cleared up.

In 3 animals 2 cc of a fourth culture were injected into another quarter 20 days later. In these cases there was a slight external inflammation, and the milk showed a few flocculi composed chiefly of polymorphonuclear leukocytes and streptococci in short chains of 4 and 6 cocci. This condition cleared up in 4 days.

A culture of a nonhemolytic *Staphylococcus aureus* was isolated from a case of mastitis. A quarter of the udder of each of 3 heifers and 2 older animals was injected with 2 cc of a 24-hour broth culture of the organism. One of the older animals, R7, was dry. The contents of her quarter became thick and the staphylococcus was recovered from the quarter 6 days after the injection. Leukocytes were present for 10 days. The udder showed no external evidence of inflammation. The other 4 animals suffered a severe general reaction. There was a marked rise in temperature, loss of appetite, stamping of feet, etc. These symptoms were evident 8 hours after injection and lasted 36 hours. The

quarters became greatly enlarged, hot and painful. The secretion from the glands was thick, viscid, fetid pus. R70 died 10 days later from a septicemia resulting from the infected quarter. The organism in question was isolated from all the tissues of the body of the animal. In the case of one animal, R46, the inflammation spread from the injected quarter to the other 3 quarters. These 3 quarters were not enlarged, but the milk contained a large number of leukocytes and nonhemolytic *Staphylococcus aureus*. The 3 adjacent quarters cleaned up in 6 days. This same animal was injected in a second quarter one month later. The animal did not show any general reaction, but the quarter was destroyed as in the first case.

Abscesses finally formed in the infected quarters of the other 3 animals. The quarters atrophied and *Staphylococcus aureus* was recovered from them until the time of slaughter 3 months later.

Two cc of a 24-hour broth culture of a 3 sugar colon isolated from the vagina of a cow was injected into a quarter of the udder of each of 3 animals. One was 10 years old, one 6 years, and the third, 3 years. In 5 hours the quarters were greatly enlarged, hot and painful. The milk was not altered in character, but there was a large number of organisms present. In 12 hours, nothing but strings of yellow viscid pus could be drawn from the quarter. Twenty-four hours later, the condition was about the same, but 48 hours after the injection there was marked improvement. The acute inflammation had subsided, and the milk from the quarter was approaching normal. There was still a larger number of leukocytes and bacteria present. In 6 days the bacteria had disappeared from the smears and in 8 days no cells or bacteria were present, with the exception of one case which showed a few cells but no bacteria. Ten days after the first injection the same amount of the same culture was reinjected into these quarters which had just recovered from the first injection. The same changes took place as the first time, but the quarters did not swell so badly and the milk did not contain so much pus. These conditions cleared up 3 days sooner than those resulting from the first injection of the colon organism.

The quarters of 2 animals, one heifer and one cow, were injected with 5 cc of a 24-hour broth culture of *Bacterium abortus*. The tissues of the quarters did not show any evidence of inflammation. In 24 hours the milk contained small clumps of cells that were visible when the milk was squirted on the palm of the hand. The organism was recovered in culture 3 days after injection. Apparently the organism did not establish itself in the udder of the animal because guinea-pig inoculations after the third day were also negative.

The quarter of one heifer was injected with 1 cc of a 24-hour broth culture of *Bact. pyocyaneum*. In 24 hours there was a slight enlargement of the quarter, and the milk contained dark yellow shreds of pus. The organism was recovered from milk 4 days after injection, but the cells did not clear up until the tenth day.

A quarter of the udder of each of 2 heifers and of one older cow was injected with 1 cc of a 24-hour broth culture of *Pasteurella bovis septica* isolated from the pneumonic lung of a calf. This caused a severe general reaction in all 3 animals. In 20 hours the temperature was elevated to 104.6, 104.2, and 103.9 F. The animals were dull, would not eat, and apparently suffered much pain. They were very uneasy, and would lie down and get up immediately. The quarters were hard and firm and bluish in color. Only a little reddish serum with a few cells could be obtained from them. The milk supply was markedly

decreased in the other 3 quarters. Rabbits injected with 0.5 c.c. of the serum obtained from the affected quarter died in 12 hours. The general symptoms subsided in 36 hours and *Pasteurella bovisepctica* was recovered from the secretion from the quarters 3 and 4 days after the injection. The affected quarters atrophied but abscesses did not form, and they remained in this condition until the animals were slaughtered.

DISCUSSION AND SUMMARY

The results show that some bacteria possess a marked degree of pathogenicity while others possess none, or give only a slight reaction which would probably occur from the introduction of any foreign fluid into the milk ducts or cisterns. One can summarize in general terms only because the resistance of the animal must be taken into consideration, and the intricate phenomenon following the introduction of foreign protein into the animal tissue is difficult to follow. The life histories of almost all of these animals have been accurately kept, and, with one exception, there is no record of the animals having suffered from infected udders up to the time the experiments were begun.

It is apparent that a certain quantity of infected milk containing a nonhemolytic streptococcus causes a more severe mastitis than the same amount of a 24-hour broth culture which contains infinitely more organisms than the milk. Whether this is due to the organism losing its virulence immediately on artificial cultivation, or whether it is due to the metabolic products formed in the milk by the streptococci, as Meyer has stated, cannot be determined definitely at this time. The milk containing the hemolytic streptococci and the cultures of the hemolytic streptococci with one or two exceptions did not cause the severe reactions that were caused by the nonhemolytic streptococci. In some cases a mild chronic infection was established, but there was not the marked difference between the ability of the milk and of the culture to produce mastitis that was observed with the nonhemolytic group.

The culture of the *Streptococcus viridans*, *Bacterium abortum* and *Bacterium pyocyaneum* produced practically the same effect on the different animals. It usually consisted of a slight swelling of the quarter infected with the formation of a few flocculi of pus in the milk, which cleared up in a comparatively short time after the injection.

The result of the injection of the organism from the colon-aerogenes group, as stated, shows that its ability to produce mastitis coincides with the work that Jensen did on organisms from this group isolated from the intestinal tract. These brought about an acute inflammation that disappeared about as suddenly as it was produced.

The nonhemolytic cultures of *Staphylococcus aureus* and *Pasteurella bovis septica* that were used produced a similar type of mastitis with general symptoms and an acute local condition followed by destruction of the quarter. The only noticeable difference was that the *Staphylococcus aureus* produced abscesses in the quarter while *Pasteurella bovis septica* did not.

Jones¹⁴ has reported finding hemolytic streptococci in the quarters that did not show an inflamed condition as well as in the diseased quarter. I did not observe this in the animals injected with the group of hemolytic streptococci or with any of the other organisms used except in the case of one animal that received an injection of *Staphylococcus aureus*. In this case not only the organism but also large numbers of leukocytes were found in the other quarters.

In most cases, after it was evident that a mastitis was permanently established, an attempt was made to improve or cure the affected quarters by injecting subcutaneously suspensions of the dead organism that had produced the conditions. This seemed to be of no value. In a number of cases suspensions of the dead organisms were injected over a period of fifteen days in gradually increased amounts. One month later there was injected a live culture of the same organism with which an attempt to immunize the animal had been made. The check animals showed no more inflammation of the udder than those receiving the dead suspensions. Milk from the infected quarters was withdrawn and injected subcutaneously. This seemed to be of no value and in some cases produced large abscesses on the animal.

It was observed that the age of the cow and the amount of milk given had an effect on the degree of mastitis produced by the organism. As a rule the cows not in milk showed no ill effects from the injection, and the same animals a few months later after calving responded to the injection according to the organism used. The heifers were much more susceptible and suffered more severely from the injection of the different bacteria used in the experiment.

CONCLUSIONS

Milk containing hemolytic and nonhemolytic streptococci from infected udders produced a more severe mastitis than 24-hour broth cultures of the same organisms when equal amounts were injected into the teat canals of healthy cows.

¹⁴ Jour. Exper. Med., 1918, 28, p. 253.

Streptococcus viridans isolated from diseased genital organs of cattle, *Bacterium abortum* and *Bacterium pyocyaneum* produced only a slight inflammation of the udder when injected into the teat canals. The mastitis cleared up in 48-72 hours after injection.

B. coli produced an acute mastitis which cleared up eight days after injection.

Staphylococcus aureus and *Pasteurella bovisseptica* produced a severe mastitis which destroyed the functional activity of the gland.

The age of the animal and the amount of milk given by the animal at the time of the injection of the various organisms are two factors which influence the degree of mastitis produced.

THE BACTERIA IN NORMAL AND DISEASED LUNGS OF SWINE

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Investigations of diseases of swine, for the past few years, have centered about 3 diseases prevalent among pigs, namely, hog cholera, infectious abortion, and infectious pneumonia. During the years 1917 and 1918 infectious pneumonia, commonly called "hemorrhagic septicemia" or "flu," increased alarmingly, and was by many thought to be associated with the human influenza epidemic of the same period. This paper deals only with the subject of pneumonia.

In reviewing the subject of pneumonia of swine it is necessary to refer briefly to the early work on a number of very similar, or similarly named, diseases, some of swine, such as hog cholera, swine plague, swine erysipelas, schweineseuche, pneumo-enterite, rouget du porc, and svin pest, as well as those of other animal species, such as fowl cholera, rabbit septicemia, rinderseuche, wildseuche, büffelseuche, and others. These diseases were recognized early as septicemias, and were collectively termed "septikämia hämorrhagica" by Hueppe.¹

The pathology as described varies widely in the different animal species, but commonly takes the terminal form of an acute respiratory infection producing pulmonary complications leading to bronchial or lobar pneumonia, hence the terms infectious pneumonia, or pneumo-enterite. In the final stages there is usually an intense septicemia, probably most conspicuous in fowl cholera and in virulent cases of swine plague, hence fowl, swine, rabbit, and cattle septicemia.

The disease was apparently recognized before the era of bacteriology by Sutton (1850), Snow (1861), Law (1875), and others. In 1876 Detmers observed an organism in the blood of swine dying of "hog cholera." This observation was later confirmed by Billings, Löffler,² and Schütz,³ and the organism is commonly called the "Löffler-Schütz Bacillus."

Preceding these observations Davaine⁴ had demonstrated a similar organism in the blood of rabbits injected with putrid ox blood. Coze and Feltz⁵ obtained similar results with putrid organic matter, and Sternberg, in 1887, isolated a like organism from the liver of a yellow-fever cadaver. Gaffky⁶ reported the

Received for publication March 13, 1922.

¹ Berl. klin. Wehnschr., 1886, 44, p. 753; 45, p. 776; 46, p. 794.

² Arbeit. a. d. kais. Gesundheitsamte, 1886, 1, pp. 46 and 546; Mitteil. a. d. kais. Gesundheitsamte, 1884, 2, p. 421.

³ Arbeit. a. d. kais. Gesundheitsamte, 1885, 1, pp. 56 and 376; Arch. f. wiss. u. prakt. Thierheilk., 1886, 12, p. 210; 1888, 14, p. 223.

⁴ Compt. rend. de l'Acad. d. sc., 1869, 68, p. 163; Bull. de l'Acad. de méd., 1872, 1, pp. 907, 976, 1004, 1100, 1234.

⁵ Recherches Experimentales sur la Presence des Infusiores et l'Etat du Sang dans les Maladies infectieuses, 1869.

⁶ Mitteil. a. d. kais. Gesundheitsamte, 1881, 1, p. 50.

isolation of the same organism from river water, and Baumgarten⁷ states that it may be found occasionally in human saliva. Perroncito,⁸ Semmer,⁹ Pasteur,¹⁰ Kitt,¹¹ and others reported the same results in studying diseases of horses, sheep, cattle, swine, and fowls.

Löffler,² Schütz,³ and Hueppe,¹ reviewing previous work, were convinced of the similarity or identity of these septicemias, and established the nature of the infection and the biologic and pathologic properties of the organisms in question. Lignières¹² studied intensively the inter-relationships of the organisms from the various animal species, and placed them in a group under the name "Pasteurella," previously proposed by Trevisan.

In the review of all of this work it may be noted that the pneumonias of the various animals are almost uniformly ascribed to pasteurella infections, the presence of other organisms being usually entirely disregarded. The same is equally true of the recent work. The possibility of the importance of other organisms in similar infections appears to have been overwhelmed by the weight of the early authorities. Birch and Benner¹³ recently recorded an outbreak of pneumonia in swine apparently due to infection with *Ps. pyocyaneus*. Murray¹⁴ has also recently described an epidemic, with a low mortality, among swine in which a small gram-negative micrococcus was constantly found. A few authors consider the possible predisposing, or secondary, rôle of other organisms.¹⁵ Aside from these few citations, and excepting "distemper" of horses and dogs, the literature on pneumonias among animals, and swine in particular, is concerned only with organisms of the pasteurella group.

The work now reported was undertaken with the object of ascertaining whether the highly infectious pneumonias so common in swine are associated solely with an infection with *B. suis*septicus, as seems to be commonly accepted in the early, as well as the recent literature. In a previous study it was found that the bacterial flora of diseased lungs was inconstant and decidedly variable, and that *B. suis*septicus was not present in sufficient numbers, nor in such percentage of cases as to warrant the sweeping conclusion that *B. suis*septicus was entirely responsible for the respiratory and intestinal lesions so common in 1918.

Arrangements were made for access to the killing floor in the pork department of one of the large Chicago packing houses. The viscera of the slaughtered pigs were observed on the moving inspecting table as they passed the Federal inspectors. The lungs were selected and tied off with a cord, severed from the other organs, and carried to one side for study and culture.

⁷ Lehrbuch der Path. Mykologie, 1888-90, 2, p. 489.

⁸ Recueil de Med. vet., 1880, 7, p. 523; Arch. f. wiss. u. prakt. Thierheilk., 1879, 5, p. 22.

⁹ Deutsch. Ztschr. f. Thiermed. u. Vergl. Path., 1878, 4, p. 244; Virchows Archiv., 1880, 82, p. 549.

¹⁰ Compt. rend. de l'Acad. des sc., 1880, 90, pp. 239, 952, 1030.

¹¹ Sitzungsber. der Gesellsch. f. Morph. u. Path. in München, 1885, 1, p. 140.

¹² Bull. de la Soc. entr. de Med. vet., 1898, 761, 797, 836; 1900, 329, 389, 469, 529.

¹³ Cornell Vet. Review, 1920, p. 176.

¹⁴ Am. Vet. Med. Assn. Jour., 1920, 57, p. 539.

¹⁵ Dimock: Ibid., 1919, 54, p. 330. Jorgenson: Ibid., p. 738. Stange: Ibid., p. 740. Salmon: Rept. Comm. of Agric., 1886, and 1888.

In order to have a basis for comparison of the bacteria of normal and diseased lungs it was decided to study the bronchial flora of 100 apparently normal lungs. For this purpose only those lungs were selected which were of a healthy pink color and which were entirely free from any visible exterior lesions. After swabs and washings had been taken the lungs were dissected and the bronchi searched for internal lesions. Occasionally lungs which were normal from the surface appearance were found to be grossly hemorrhagic on sectioning, or showed lesions along the mucous surface of the bronchi. Such lungs and the corresponding cultures were not included in the "normal" series.

Having determined, by the study of these 100 normal lungs, the presence of a "normal" flora, the study of pneumonic lungs was carried out on 314 lungs showing various degrees of congestion, consolidation, or adhesions.

Technic.—The upper lobe of the normal lungs was selected as the point most remote from possible contamination, and inoculations were made from this region when possible. Such selection was naturally impossible in many cases of pneumonia.

The lung surface was swabbed with 10% phenol, then cut transversely midway between the tip of the lobe and the junction of the bronchi and trachea. The cutting was continued until the bronchus was exposed. The surface of the bronchus was further exposed by scraping, after which it was swabbed with phenol and then severed with a sterile scalpel. Sterile swabs were then introduced, then the bronchial secretions were washed loose and pipetted out in sterile saline for plate inoculations.

The swabs were taken at once to the laboratory where they were stroked on plain and blood-agar plates, and the washings also plated on Endo medium as a control of contamination. Selected colonies were replated and pure cultures isolated for identification on plain or blood-agar slants.

Owing to the proposed extent of the study examination was restricted to those organisms which were constantly present in significant numbers. Since this study was devoted to pneumonia the pneumococcus-streptococcus and the pasteurilla groups were regarded as probably of greatest importance, while earlier experience pointed to the *B. suispestifer* group as of possible significance.

Sections of the pneumonic lungs enclosing the consolidated portions were cut out and wrapped in sterile paper and carried to the laboratory.

Here the lung surface was carefully seared, cut, and cultures taken from the bronchi. It was decided in the work on normal lungs that the use of plain agar added nothing of importance to the findings, and after the first 30 examinations only blood-agar and Endo medium were used.

Material was cut and fixed for future sectioning in 10% formalin. The tissues were imbedded in paraffin, and the pathologic study and correlation with the bacteriologic findings will form the basis of a later paper.

Bacteriology of Normal Lungs.—In the tables the term “pure culture,” as used, includes not only strictly pure cultures, but also those which were pure with the exception of an occasional obvious contaminating colony. The term “many colonies” refers to the presence of the organism in predominance and in numbers sufficient to warrant attributing some significance to its presence. The term “few colonies” was applied to the occasional finding of from a single colony up to 10 or 20, a condition differing sharply from those cultures yielding “many colonies.” Just what degree of importance is to be attributed to the presence of these organisms in small numbers may be shown by the pathologic study, when it will be possible to compare the bacterial findings with the type and stage of the particular pneumonia in question.

Only 3 groups of organisms were isolated in pure culture with any degree of frequency in this study. The incidence of these pure cultures is recorded in table 1.

TABLE 1
COMPARISON OF BACTERIA FOUND IN PURE CULTURE IN APPARENTLY NORMAL AND PNEUMONIC LUNGS

	Normal Lungs (100 Specimens)		Pneumonic Lungs (314 Specimens)	
	Number of Specimens Yielding Culture	Percentage of Specimens Yielding Culture	Number of Specimens Yielding Culture	Percentage of Specimens Yielding Culture
<i>B. suis</i> septicus, pure culture.....	2	2	138	44
Inulin-fermenting streptococcus, pure culture.....	0	0	91	29
<i>B. alkaligenes</i> type, pure culture....	1	1	1	0.3

Examination of table 1 shows that only 2 of the 100 apparently normal lungs yielded pure cultures of *B. suis*septicus. A single pure culture of a *B. alkaligenes* type was found. The inulin-fermenting streptococcus was not found in pure culture in any normal lung. In addition to the pure cultures isolated, the 3 significant organisms were

frequently found in mixed culture. The total incidence of these 3 types, including both pure and mixed culture, is recorded in table 2.

In addition to those organisms present in considerable numbers, various streptococci were commonly found in smaller numbers, together

TABLE 2
COMPARATIVE INCIDENCE OF THE SIGNIFICANT ORGANISMS FOUND IN APPARENTLY
NORMAL AND PNEUMONIC LUNGS

Organisms	Relative Frequency on Original Plating	Normal Lungs (100 Specimens)		Pneumonic Lungs (314 Specimens)	
		Number of Specimens Yielding Culture	Percentage of Specimens Yielding Culture	Number of Specimens Yielding Culture	Percentage of Specimens Yielding Culture
B. suisepiticus.....	Many colonies	2	2	123	40
	Few colonies	2	2	45	14
Inulin-fermenting streptococcus	Many colonies	5	5	93	29
	Few colonies	7	7	18	6
B. alkaligenes type.....	Many colonies	1	1	6	2
	Few colonies	2	2	4	1
			3		3

TABLE 3
COMPARATIVE INCIDENCE OF STREPTOCOCCI IN APPARENTLY NORMAL AND PNEUMONIC LUNGS

	Normal Lungs			Pneumonic Lungs		
	Number of Cultures Isolated	Number of Specimens Yielding Cultures	Percentage of Specimens Yielding Cultures	Number of Cultures Isolated	Number of Specimens Yielding Cultures	Percentage of Specimens Yielding Cultures
Streptococci present, usually but 3-5 colonies.....	..	89	89	..	183	60
S. pyogenes.....	1	1	1
S. faecalis.....	19*	13*	13	11	9	9
S. mitis (type).....	23	17	17	14	12	4
S. mitis (var. inulin +).....	14	12	12	114	111	34
S. salivarius.....	8	6	6	13	10	3
S. non-hemolyticus iii.....	12	8	8	11	8	3
S. non-hemolyticus iii.....	4	3	3
S. equinus.....	33	22	22	27	21	7
S. ignavus.....	9	6	6	8	7	2

* In many instances 2 or 3 apparently different colonies from the same specimen proved culturally identical.

with other organisms. An attempt was made to isolate one of each type of streptococcus colony found on the original blood-agar plates. These cultures were then subjected to the Holman system of classification with the results shown in table 3.

The figures in table 3 indicate the relative incidence of the various types rather than the specific incidence. The important point brought out in the table is the isolation of an inulin-fermenting streptococcus from 12% of the apparently normal lungs examined. The characteristics and possible significance of its presence will be discussed later.

Cultural Characteristics.—The biologic and morphologic characteristics of *B. suis* are too well known to require further description. The point of significance, as indicated in this study, is the isolation of the organism from only 4% of the apparently normal lungs. It is commonly stated that *B. suis*, in an avirulent form, is widely distributed in the lungs of normal swine, and that such animals serve as foci of infection under proper conditions. Such a condition is certainly not indicated by the results of these observations. In two cases *B. suis* was recovered in pure culture in large numbers, and, while the lungs were apparently normal, the possibility of an impending pneumonia cannot be overlooked. In only two other cases was the organism found in small numbers, under conditions which might warrant the assumption of the status of a normal carrier.

Two organisms other than *B. suis* were isolated early in the study of normal lungs. One of these grew on Endo medium, producing typical paratyphoid colonies, but failed to ferment any of the sugars tested, and was regarded as a *B. alkaligenes* type. The other was a diplococcus, or short-chain streptococcus, which developed more or less pneumococcus-like colonies on blood-agar; produced a distinct green halo without complete hemolysis; coagulated milk; fermented inulin; did not have a demonstrable capsule; was not soluble in bile; and was not agglutinated by pneumococcus type 1, 2 or 3 antiserum. Neither of these two organisms were frequent in the normal lungs, but they were conspicuous by their peculiar reactions.

In addition to these organisms several other common types were rather constant, although present only in too small numbers to be of significance, and never in pure culture. *Staphylococcus albus* was isolated from 49 lungs, there being in most cases only 2 to 4 colonies on the original plates. In two instances there were from 25 to 30 colonies, together with a variety of other organisms. *Staphylococcus aureus* was found in 15 cases, but never more than 4 or 5 colonies from one specimen. A large whitish colony, which produced a broad hemolytic zone, was observed in 12 instances. The organism was a tiny gram-positive, sporing bacillus. Never more than 1 or 2 colonies were found

on any one plate, and this organism was regarded as a chance contamination, probably of mouth origin. A gram-negative diplococcus which produced waxy-mucoid colonies and closely resembled *Micrococcus pharyngis siccus*, was found in 12 cases, but never in significant numbers. One other type of colony was frequently encountered—in 38 instances. This was a large spreading, green colony, surrounded by a broad, diffuse zone of incomplete hemolysis. This organism also was a small, gram-positive, sporing bacillus, which produced a strong odor of ammonia on blood-agar plates. There were usually only 1 or 2 colonies, rarely 3 or 4, on any one plate, and it also was regarded as a contaminating organism, probably of mouth or fecal origin. This list comprises all organisms found in any degree of frequency under the conditions of this study.

Five specimens did not yield any growth on either blood agar or Endo medium.

Pathogenicity of Bacteria from Apparently Normal Lungs.—Saline washings from the bronchi of normal lungs were injected intraperitoneally into 18 white mice. One c c of a turbid emulsion of mucus and the contained bacteria was injected into each mouse. None of the mice died, and only 3 showed any visible reaction, but these were normal on the third day after inoculation when they were killed. Stained smears from the peritoneal cavities did not reveal any bacteria. It was evident that there were no organisms in these specimens with any degree of pathogenicity for white mice.

Six strains of *B. suis*-like organisms were tested for virulence on rabbits by injecting subcutaneously 0.5 c c of a saline suspension of a 24-hour blood-agar culture. Two strains produced no evident reaction, and further study of the fermentative ability showed that, while similar, they were entirely distinct from *B. suis*. Two typical strains of *B. suis* were observed in large numbers in pure culture on the original plates, while 2 other strains were present only in small numbers. The 4 rabbits inoculated with these strains all died within 3 days, and the typical bipolar organisms were found in abundance in the heart blood of all.

One strain of the *B. alkaligenes* type was inoculated into a rabbit with no visible reaction.

The pathogenicity of the inulin-fermenting streptococcus from normal lungs was not tested, but will be discussed later in connection with the study of the pneumonic lungs.

Bacteriology of Pneumonic Lungs.—In view of the results obtained from the apparently normal lungs attention was focused on the three bacterial types emphasized in the foregoing. The secondary organisms listed appeared in approximately the same numbers in the plates from the pneumonic lungs, but in considering such a large number of specimens it was found impossible to study these organisms intensively. The streptococcus group, however, was studied for comparison with the incidence of the inulin-fermenting type. According to the Holman classification they were grouped as noted in table 3.

In comparing the streptococci from the normal and pneumonic lungs it would appear, at first sight, that there are relatively fewer streptococci, other than the inulin-fermenting type, to be found in the pneumonic lungs. These results are undoubtedly due to the preponderance of the inulin-fermenting type, leading to the overlooking of the other types which were always few in number. A careful study of this particular point would probably show an approximately equal incidence of the streptococci, excepting the inulin-fermenting strain, in both normal and pneumonic lungs.

Two striking facts may be observed by comparison of the bacteria found in normal and pneumonic lungs, as shown in table 1: First, pure cultures of *B. suis* were found in 44% of pneumonic lungs, but in only 2% of normal lungs. Second, pure cultures of the inulin-fermenting streptococcus were found in 29% of the 314 pneumonic lungs, but in none of the normal lungs.

It is evident, from perusal of the tables, that there are only two groups of organisms which are consistently present in numbers sufficient to warrant closer study:

B. suis was recovered from 4% of the 100 normal lungs examined. Of these 4% only 2% were present in pure culture.

B. suis was recovered from 54% of all pneumonic lungs examined. Of these, 54% pure cultures were found in 44%, while in 10% more it was present in mixed culture and in variable numbers.

The inulin-fermenting streptococcus was isolated from 12% of the normal lungs. It was not found in pure culture in any of the normal lungs.

The inulin-fermenting streptococcus was isolated from 35% of the 314 pneumonic lungs examined. In 29% of the cases it was present in pure culture, while in 6% more it was observed in varying numbers together with other organisms.

In 58 cases, or 15% of the pneumonic lungs, the two types of organisms occurred together in large and approximately equal numbers. The pathology of such cases will constitute a topic for special study later.

Description of the Inulin-Fermenting Streptococcus.—In classifying this organism according to the Holman system it falls within *Streptococcus mitis* group. Because of the ability to ferment inulin it would be classed as a variety within the “*mitis*” group. The occurrence of such a variety has been noted, and Holman¹⁶ says: “I believe that the inulin variety of streptococcus exists, and that it may be more common than I have indicated.”

The organism is typically a diplococcus when stained in bronchial mucus smears; ovoid to almost lanceolate at times; never in long chains in the bronchial secretions, although at times in short chains of 6 to 8 cells, but always distinctly in pairs in such cases; commonly found in clumps of typical gram-positive diplococci; capsules, or even doubtful indications of such, have never been demonstrated.

The first generations on blood-agar are commonly very pleomorphic; some strains maintain this character indefinitely, while others seem to revert quickly to the typical morphology. Such pleomorphic strains show bacillary forms of all conceivable types, together with typical diplococci and chains of pairs of varying length. These strains also display a great deal of gram-negative material, both as organized cells and as cells in all stages of decomposition.

Serum broth and milk appear to offer more favorable conditions for growth of the earlier generations, the cultures in these mediums usually displaying less pleomorphism, and conforming more to the original type, except in the tendency toward chain formation, which is more pronounced here than in cultures on blood agar. These chains uniformly show a distinct arrangement of the cells in pairs.

Cultural Characteristics.—The reaction on blood agar is quite constant for the entire group of 128 strains from both normal and pneumonic lungs. The colonies at 24 hours are about 0.5 mm. in diameter, on the average, tending to remain discrete, round, entire edged, slightly raised center, yet no tendency to form the typical nipped streptococcus colony except on prolonged incubation on dried plates, when a slight nipple formation may appear. Old colonies reach a maximum size of about 2 mm.

¹⁶ Jour Med. Research, 1916, 34, p. 388.

The colonies are uniformly surrounded by a distinct zone of green. True hemolysis does not develop even after prolonged incubation. Thirty strains were plated at one time on blood agar in order to compare the colony characteristics; while there appeared some slight individual variations there was a great similarity, approaching identity, of all strains.

The reaction in milk was identical for all strains. An acid reaction was apparent at 24 hours, together with decolorization of the litmus indicator in the lower half of the tube; coagulation appeared quite constantly at 48 hours, with a few strains showing a tendency to coagulate at 24 hours.

These tests were run in serum-sugar broth with the Andrade indicator. Pig serum water (1 part serum to 3 parts distilled water) was added to veal infusion broth (1 part serum water to 4 parts broth; final reaction 7.6 to 7.8). The mannite and lactose broth contained 1% of the sugar, while the inulin and salicin broths were made up to only 0.1%. A few early tests were run in 1% salicin and inulin, but some of the results with salicin suggested a possible inhibition, and the use of the smaller amount was adopted.

The reactions in the 4 sugar broths were very constant. The characteristic reaction was acid and there was coagulation at 24 hours in the lactose, salicin, and inulin serum broth, with no perceptible change in the mannite broth. Some of the freshly isolated strains gave doubtful, and occasionally negative, reactions in one or more of the sugars. Repetition of the test resulted, in almost every case, in a typical completely positive reaction, while in other cases the acid reaction was evident but did not progress to the point of coagulation. Correlation of these atypical fermentative results with poor growth or pleomorphism was in most instances demonstrable by staining.

Nine strains from normal lungs and 102 strains from pneumonic lungs were tested for solubility in bile. None of the strains showed the slightest trace of solubility, while control tests with *Pneumococcus* type 1, 2 and 3 were always completely positive.

Serologic Study.—Agglutinative typing was begun. Thus far only a single serum has been tested, and further study on the direct agglutination and agglutinin absorption will be reported later. Sixty-three strains were tested against the single serum, and 19 agglutinated to the full titer of the serum, while the remainder were completely negative. The results of these tests with a single serum indicate that the strains will fall in at least 2 agglutinative groups.

No particular difficulty was experienced in making the tests, as these strains were all diplococci or short-chain streptococci in broth culture, and did not show the slightest tendency toward spontaneous agglutination. The organisms were grown for 24 hours on blood-agar slants, then 3 to 4 c c of sterile 0.2% glucose broth were added aseptically and the cultures were incubated another 24 hours. Heavy suspensions were obtained, which were diluted with formaldehyd-salt solution.

Pathogenicity.—These organisms, which appear to have highly invasive powers in pigs, are not markedly pathogenic for white mice or rabbits. Two mice were inoculated intraperitoneally with 0.5 c c of a saline emulsion of purulent bronchial mucus which yielded many colonies of the inulin-fermenting streptococcus. Neither mouse died. Ten mice were inoculated with suspensions of blood-agar cultures or with serum broth cultures. Three of the 10 mice died. Two of the 3 showed only a *B. proteus* type of spreader present in the heart blood and peritoneal fluid, with no streptococci demonstrable either by strain or culture (the third mouse was killed by the others). Two others of the 10 inoculated were markedly affected, but recovered, while the other 5 showed no visible reaction.

A rabbit was given 1 c c of a heavy suspension of a blood-agar culture intravenously. No perceptible reaction was noted, and the animal was repeatedly injected with live cultures for the production of the antiserum used in the agglutination tests.

SUMMARY

This paper reports the bacteriologic study of 100 apparently normal lungs of pigs and 314 lungs which showed pneumonic lesions of varying extent and intensity.

Three organisms of possible significance were isolated from the normal lungs: *B. suis* was found in 4% of the 100 specimens; in 2% it was present in pure culture and in large enough numbers to suggest the possibility of an impending pneumonia; in the other 2% there were only a few colonies of this organism together with a mixture of other bacteria. A nonfermenting, typhoid-like, bacillus, considered a *B. alkaligenes* type, was found in pure culture in 1 case, and in mixed culture in 2 other cases. An inulin-fermenting streptococcus was isolated from 12% of the cases; in 5 cases there were many colonies present, while in the other 7 cases there were a few colonies together with other organisms; no pure cultures were observed.

A variety of other organisms, particularly streptococci, were observed in small numbers, and always in mixed culture.

B. suis was found in pure culture in 138 cases, or 44% of pneumonic lungs examined. It was found in small numbers mixed with other organisms in 30 more cases, or 10%, making a total of 168 specimens, or 54% of those examined, from which the organism was isolated.

The inulin-fermenting streptococcus was found in pure culture in 91 cases, or 29%. In an additional 6% of specimens it was present in mixed culture in small numbers, making a total of 111 specimens, or 35% of those examined, from which the organism was isolated.

No other organisms appeared in pure culture, nor in mixed culture in sufficient numbers to indicate any etiologic significance.

The 2 strains, *B. suis* and the inulin-fermenting streptococcus, were found in pure culture, or together in approximately equal numbers, in 63% of the 314 specimens examined. A study of the imbedded tissues is being made and will be reported later.

It appears to be generally accepted that the mere presence of *B. suis* constitutes sufficient evidence as to its etiologic significance in swine pneumonias. Judged by the same criterion, the streptococcus here described would seem to be of almost equal importance.

Agglutination tests with a single serum indicate that there are at least 2 types within the group of 128 strains of the streptococcus studied. Further work is in progress and will be reported later.

THE LOCAL INFLAMMATORY REACTION PRODUCED BY THE TETANUS BACILLUS

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A review of recent literature and of standard books of pathology has not revealed a description of the reaction of the tissues to the presence of the bacillus of tetanus. Therefore the experimental production of tetanus in guinea-pigs was undertaken to study the reaction of the tissues at the site of inoculation to the tetanus bacillus.

In the production of the disease were used pure cultures of a strain of *B. tetanus* known to be virulent to guinea-pigs. This strain was secured from Dr. E. M. Houghton of Parke, Davis & Company of Detroit. Injections were made of actively growing cultures 72 hours old, so that few if any spores were introduced. Injections of cultures in fluid medium produced almost immediate symptoms because of the toxin present, and this method had to be abandoned and injections made of suspensions in salt solution of tetanus bacilli grown on the surface of solid medium. The cultures were incubated in a chamber of hydrogen gas; later a chamber freed of oxygen by pyrogallie acid and sodium hydrate was substituted. The dose was 0.25 c c of a suspension of standard turbidity and of sufficient strength to produce tetanus in guinea-pigs of 250 to 300 gm. The sites for the injections were the subcutaneous and muscular tissues of the right thigh and also the foot pad. In the former some of the bacilli reached the thigh muscles so that muscle cell reactions could be observed, and in the second the site of local disease was the same as the site of inoculation frequently encountered in tetanus in human beings. Studies were made of serial microscopic sections of the lesions, made at intervals from 12 hours after inoculation up to several days after the appearance of generalized tetanus.

All animals from which lesions were studied presented symptoms of tetanus in nearly all instances as early as about 12 hours after the injection. A definite rigidity of the muscles of the injected extremity, the so-called "local tetanus," was observed first. After a few hours the

opposite extremity commonly became rigid and finally the whole body was involved—"ascending tetanus"—with opisthotonus, convulsions, and trismus.

Microscopic examination in the early stages of the disease, that is about 12 hours after the inoculation, revealed an intense cellular invasion of the affected area. The site of local reaction was pretty sharply defined by the cellular invasion. Definite necrosis or abscess formation

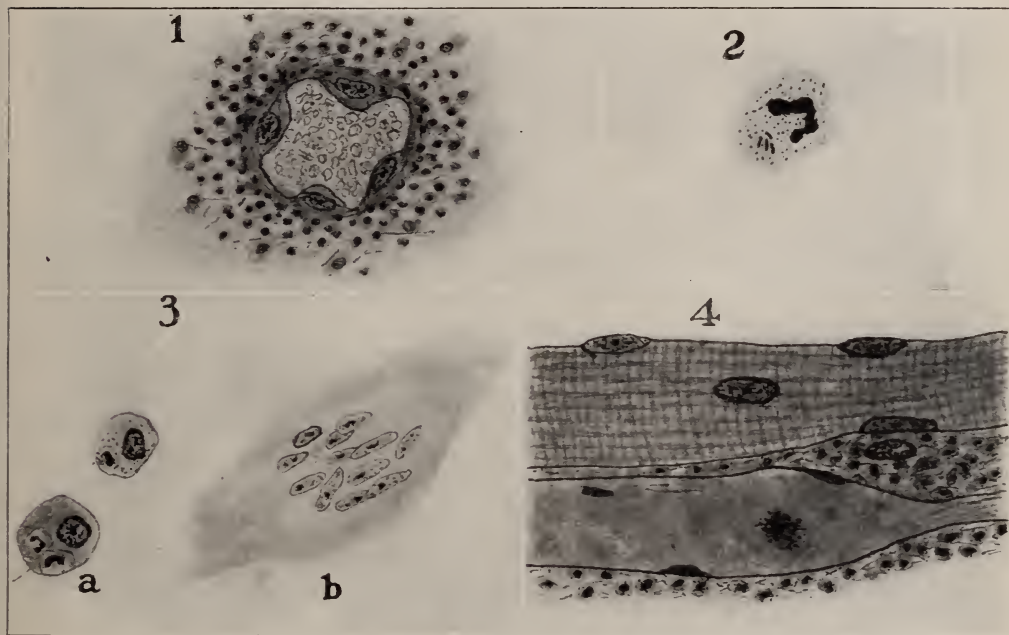


Fig. 1.—Hyperplasia of endothelial cells in the capillary wall.

Fig. 2.—Polymorphonuclear leukocyte with four tetanus bacilli.

Fig. 3.—Leukocytes engulfed by mononuclear cell (a) and giant cell (b).

Fig. 4.—Disintegration of nucleus of muscle cell and waxy change of muscle (van Gieson stain).

did not develop. The predominating cell in the early stage was the polymorphonuclear leukocyte which makes up about 80 to 90% of the cells present. In addition a few mononuclear leukocytes and large mononuclear wandering cells are present. The capillaries are engorged and the endothelial cells are enlarged so that they project into the lumen of the vessel (fig. 1). There is also a distinct perivascular infiltration of inflammatory cells. The bacilli occur chiefly in clumps

scattered in the spaces between the leukocytes and fixed cells. A few bacilli, however, are seen within polymorphonuclear leukocytes (fig. 2) indicating that tetanus bacilli exert a positive chemotaxis, which is in agreement with Werigo¹ who doubted that any virulent bacteria would exert a negative chemotaxis on leukocytes, as claimed by some and recently by Metalnikow.² Some of the polymorphonuclear leukocytes are engulfed by large mononuclear wandering cells, as shown in fig. 3. At this stage no hyaline or waxy change has taken place in the muscle, but the nuclei in the sarcolemma are swollen. No foreign body giant cells and no plasma cells are present, and there is no hyperplasia or increase of fibroblasts in the early stages. The alveolar tissue is invaded by large numbers of cells as is the loose fibrous tissue between the muscle bands, so that the latter are widely separated.

In the older lesions polymorphonuclear leukocytes become less numerous while lymphocytes and mononuclear wandering cells increase so that they make up from one-half to one-third of the cells present. In the later stages, that is, after 5 or 6 days, large fibroblasts are present and the reparative phase of the reaction is now established. The muscle cells are also affected as evidenced by disappearance of the striations, granular disintegration of nuclei (fig. 4), and the presence of a well defined waxy degeneration. The formation of giant cells is another characteristic of the later stages of the reaction; they mingle with other cells, and their nuclei are centrally located (fig. 3). Plasma cells have not been found in any of the microscopic preparations of lesions of even several days' duration.

In connection with these observations, microscopic studies were made also of the local lesion in the sole of the foot obtained, through the courtesy of Dr. E. R. LeCount, postmortem from the body of a man who died from tetanus. While no conclusions are drawn from this single examination of human material, it may be noted that the cellular invasion, the hyperplasia of fibroblasts, and the inflammatory reaction generally corresponded in all essentials to the changes observed in the older experimental lesions.

SUMMARY

When typical tetanus is produced in guinea-pigs by injection into the thigh muscles and foot pads of suspensions of tetanus bacilli, an

¹ Ann. de L'Inst. Pasteur, 1894, 8, p. 1.

² Ibid., 1921, 35, p. 363.

intense inflammatory reaction promptly ensues at the site of inoculation, in which both wandering and fixed cells take part.

The tetanus bacillus exerts a positive chemotaxis and is taken up by polymorphonuclear leukocytes; it also induces the formation of giant cells.

In one human case the local reaction to the tetanus bacillus corresponded in all essentials to the reaction of experimental inoculation in guinea-pigs.

ON THE BIOLOGIC PROPERTIES OF PATHOGENIC MOLDS *

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The biologic properties of the higher fungi are almost totally ignored in most works dealing with mycology and particularly bacteriology, despite the fact that their proteolytic ferments, at least, have been made the object of numerous studies. Attempts to classify these organisms along these lines have, to date, failed; yet these properties, although not of practical value, are at least of value from a scientific point of view.

It might be well to remark here, that after 18 months' study of the growths of ringworms, obtained from Dr. Sabouraud, on various culture mediums of American made ingredients, we have, with other investigators, come to the conclusion that for the present, recognition of any of these organisms depends on their characteristics developed on mediums made with the French ingredients and methods used by Dr. Sabouraud. If Dr. Sabouraud's studies, descriptions, and methods, all of which depend on culture mediums, whose ingredients are difficult to obtain in America, are to be accepted finally as a basis for the classification of the pathogenic molds, then it appears that only by studies along these lines can we arrive at a definite conception of the pathogenic ringworm flora of America, unless the all too difficult problem of French products or their exact chemical nature and p_H be solved by the use of American produced substances.

So far as I have been able to determine, the studies undertaken in this paper have been limited to few fungi and generally to the most important of their ferments, namely, gelatinase. Is this proteolytic property possessed by all of the higher vegetable parasites, and what is the relative activity of that possessed by each of them. Is this ferment soluble or insoluble in all cases, and to what extent?

In 1895, MacFadyen¹ definitely showed that the ringworm fungus produced a ferment capable of digesting gelatine. Although he worked

Received for publication March 17, 1922.

* Aided by funds accruing from the sale of Arsphenamine.

¹ Jour. Path. & Bacteriol., 1889, 33, p. 176.

with fresh specimens of the trichophyton, he made no note of the varieties used. However, MacFadyen not only determined that the trichophytos he used possessed proteolytic powers, but that the enzyme was soluble and given off by the fungus as was indicated by the digestion of fresh gelatine when liquefied fungus-free digested gelatine was added to it. He further demonstrated that a temperature of 100° C. for 2 minutes destroyed this ferment, and that acids retarded, whereas alkalis accelerated, its action.

Roberts² showed that even 6-year old specimens of fungi retained this property, and came to the following conclusions:

1. That the active agent is contained in the mycelium itself and is not formed outside the body of the parasite by secondary changes in the medium.

2. When the fungus has been preserved in a perfectly dry state, that this active principle can retain its activity in a dormant state for a period of 6 years.

3. That the activity of the agent is independent of the life of the fungus.

4. That small amounts of the active agent can digest comparatively large amounts of gelatine.

5. That this active agent is in the nature of a soluble ferment.

6. That the activity is destroyed by heat at a temperature of 85 C. for 2 minutes.

Like MacFadyen, Roberts drew his conclusions from a study of a limited number of these organisms. Most of his work remains exact. It is interesting to note that as a result of his studies as well as those of Ruffi, the proteolytic property of these parasites have no relation to their virulence since it is present in dead fungi also. And the latter fact is true, not only for the limited number of organisms studied by him, but likewise for all of the organisms studied in this paper.

This study has for its objects:

1. A study of the proteolytic ferments of 26 pathogenic fungi.
2. A study of the amylotic ferments.
3. Their action on various sugars and on litmus.
4. A study of indol production.
5. A study of the toxins of several species.

PROTEOLYTIC FERMENTS

The following table gives the organisms studied and their comparative proteolytic properties in 10 cc of 10% gelatin. Plus 3 is used to

² Brit. Med. Jour., 1899, 24, p. 13.

indicate complete liquefaction in 24 hours; plus 2, a moderate liquefaction for the same period of time; plus 1, a slight liquefaction; and —, none at all. Since all these organisms are particularly sensitive to changes in heat, light, moisture, etc., the experiments were conducted under as uniform conditions as possible.

ORGANISMS STUDIED AND THEIR COMPARATIVE PROTEOLYTIC PROPERTIES IN 10 Cc OF 10% GELATIN

Organism	Liquefaction in 24 Hours
<i>Tricophyton niveum radicans</i>	Plus 3
<i>Tricophyton vinosum</i>	Plus 3
<i>Microsporon pubescens</i>	Plus 3
<i>Achorion quincekeum</i>	Plus 3
<i>Tricophyton violaceum</i>	Plus 2
<i>Tricophyton fumatum</i>	Plus 2
<i>Tricophyton laticolor</i>	Plus 2
<i>Sporotrichum beurmanii</i>	Plus 2
<i>Sporotrichum schenkii</i>	Plus 2
<i>Tricophyton crateriform</i>	Plus 1
<i>Tricophyton acuminatum</i>	Plus 1
<i>Tricophyton sulphureum</i>	Plus 1
<i>Tricophyton exsiccatum</i>	Plus 1
<i>Tricophyton cerebriform</i>	Plus 1
<i>Tricophyton plicatile</i>	Plus 1
<i>Tricophyton gypseum asteroides</i>	Plus 1
<i>Tricophyton granulosum</i>	Plus 1
<i>Microsporon lanosum</i>	Plus 1
<i>Tricophyton roseaceum</i>	—
<i>Microsporon audouinii</i>	—
<i>Microsporon fulvum</i>	—
<i>Sporotrichum gougeroti</i>	—
<i>Actinomyces bovis</i>	—
<i>Achorion schoenleinii</i>	—
<i>Achorion gallinae</i>	—

The ring-worm fungi in this group, however, showed liquefaction at a later stage.

SOLUBILITY OF THE FERMENT

Truffi, as well as Roberts, showed that liquefied gelatine was able to hold and transmit this proteolytic body, but this is certainly not true of all the fungi. All the organisms in this study did not exhibit this property when filtered liquefied gelatine was used, and we might therefore conclude that gelatinase in them is not a soluble one. Fresh gelatine in contact for 72 hours with liquefied gelatine obtained with *Microspora audouinii* and *lanosum*, *Tricophyton laticolor*, *Achorion quincekeum* and *Sporotrichum* (*beurmanii* and *schenkii*) showed no liquefaction at all with the parasites just noted.

Second, this special enzymotic power, as present in liquefied gelatine, varies definitely in strength with the organism. Whereas a certain number will liquefy the fresh gelatine in 24 hours when the same

amounts of liquefied material are used (*vinosum*, *pubescens*, *niveum* *radians*), others require 48 hours to complete it (*plicale*, *cerebriform*, *sulphureum*).

Summary.—Gelatinase is a common property of the higher vegetable parasites, and varies with the organism, but not so much because of the rapidity with which that organism grows, since *Tricophyton gypseum asteroides* is a "quick grower," in mycologic parlance, and yet caused but slight liquefaction at the end of 24 hours. It is a question of quantity or quality of gelatinase, or both. Furthermore, the solubility of this ferment varies with the organism.

AMYLOLYTIC FERMENTS

Both MacFadyen and Roberts, as well as Bodin,³ working with *Achorion gypseum*, were unable to demonstrate an Amylolytic ferment. We were unable to detect sugar with Benedict's solution at any time during the 2 months of their growth in a starch-water medium. They grow quite well in 3 and 5% starch solutions, indicating that they can assimilate the larger starch molecule without previous hydrolytic changes.

SUGAR FERMENTATION AND LITMUS REACTION

For this study a Russell's modified double sugar medium prepared with lactose and glucose, as usual, was used, and a series of tubes each prepared with one only of the following: lactose, glucose, maltose, saccharose, dextrin, and levulose, and with end result adapted for fungi.

The effects of each fungus on one double sugar and one single sugar medium were studied for color changes and gas production.

It may be noted that all the fungi studied grew quite well on these mediums, but most lacked the characterization given them by Sabouraud's concentrated and proof mediums.

None of the organisms listed below altered litmus or caused fermentation of any of the sugars: *Tricophyton*, *acuminatum*, *crateriform*, *violaceum*, *fumatum*, *sulphureum*, *exsiccatum*, *cerebriform*, *plicatile*, *asteroides*, *granulosum*, *lacticolor*, *radians*, *rosaceum*, *vinosum*, *ochraceum*; *Microsporon*, *audouini*, *pubescens*, *fulvum*, *lanosum*; *Achorion*, *schoenleinii*, *gallinae*, *quinckeanum*, *gypseum*.

These organisms were also grown on the same medium without peptone, but did not, under these circumstances, ferment any of the sugars used.

³ Ann. de Dermat., 1907, 8, p. 585.

INDOL PRODUCTION

Böhme's technic ⁴ was used. The organisms were planted in sheep serum broth. All the implants did not grow, but in none of those that grew, were we able to detect indol.

TOXIN PRODUCTION BY PATHOGENIC FUNGI

Cutaneous affections caused by the higher vegetable organisms are ordinarily not associated with systemic disturbances indicative of intoxication, and few among the numerous investigators have been able to demonstrate the presence of toxic substances elaborated by them.

As the work of Plato, Block, and Massini ⁵ has shown, the dermatophytous do produce toxins. Further, toxic soluble substances have been shown to be elaborated by *Oidium albicans* (Charrin, Ostrowsky, Roger, Concetti quoted by Brumpt), by *Discomyces* and some species of *Aspergillus*. That they really have a toxic influence is shown by the destruction of bone by the ray fungus, the abscesses in sporotrichosis, blastomycosis, kerion and folliculitis agminata.

In our work 5 organisms were studied: *Tricophyton acuminatum* and *gypseum-asteroides*; *microsporon audouini*, *sporotrichum beurmanii* and *achorion schoenleinii*.

These fungi were cultivated in a proof-medium with bouillon as the base, and sealed to prevent evaporation. The tricophytos and sporotrichum were grown for 8 weeks; the microsporon and achorion, for 14 weeks. At the end of these periods, these were filtered, after thoroughly breaking up the growth, first through sterile filter paper, then through a sterile kitasato candle. A control flask of this medium was filtered in the same manner.

Eight ounce guinea-pigs were injected intraperitoneally with 2 c c of the control and 2 c c of each filtered culture. The pigs apparently suffered no untoward reaction. One week later, 4 c c of each medium were used. The microsporon, sporotrichum, and the control gave negative results, whereas the pigs injected with the tricophytos and achorion died. That injected with *T. acuminatum* succumbed in 24 hours; that with *T. gyp. asteroides*, 10 days later, and that with achorion, 48 hours later.

After death there was found mainly a marked suprarenal vascular injection. The appearance resembled that produced by the intraperitoneal injection of diphtheria toxin. Cultures made from the peritoneal fluid remained negative.

⁴ J. Am. Med. Assoc., 1921, 77, p. 959.

⁵ Cited by Sabouraud, Les Teignes, 1902, p. 735 and 736.

Sabouraud⁶ states "That many writers have inoculated the elaborated products of cultures, but that the resultant effects were due to the toxic action of the peptones." Inasmuch as the control pig suffered no ill effects, it seems natural to conclude that the trichophytons and the achorion schoenleinii do elaborate toxic products.

CONCLUSIONS

A proteolytic ferment is common to all the pathogenic molds studied, but its solubility varies with the organism.

They possess no amylotic properties.

They do not ferment saccharose, dextrin, glucose, levulose, maltose or lactose, and they produce no acids or bases.

The trichophytons (*Trichophytons acuminatum* and *gypseum-asteroides*) and *Achorion schoenleinii* elaborate toxins fatal to guinea-pigs.

⁶ Les Teignes, 1902.

ON SPECIFIC ERYTHROPRECIPITINS (HEMOGLOBIN PRECIPITINS?)

LUDVIG HEKTOEN AND KAMIL SCHULHOF

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In 1901 Leblanc reported that beef hemoglobin acted as a specific precipitinogen, and this discovery was confirmed by Demees as well as by Ide, in whose laboratory the work was done.¹ Klein² found that injection of rabbits with watery extracts of red corpuscles gave rise to specific precipitins and Leers³ confirmed while others either denied or doubted that specific erythroprecipitins could be produced.³ Consequently, it seemed advisable to make new experiments, and we first studied precipitin formation in response to extracts of various corpuscles (table 1) on the same general plan as Klein and Leers.

The extracts are made by suspending the carefully washed corpuscles of a definite quantity of blood in many times that quantity of sterile distilled water, then adding as much again of 1.8% salt solution and centrifugating thoroughly. In this way clear solutions with 0.9% of sodium chloride are obtained; to insure sterility they may be passed through Berkefeld filters, which also removes the stromata and perhaps limits the number of antigens in the filtrates. In most cases the solutions we use are made so that 50 c c contain the extract of the corpuscles in one c c of blood. Five or 6 injections are given rabbits intravenously every 3 days, beginning with about 2 or 4 c c and increasing by about 2 c c each time. As usual, the precipitin content of the serum seems to reach the highest point 7-8 days after the last injection. The tests are made with progressive dilutions of erythrocytic extracts or serum in small tubes, a small quantity of precipitating serum being introduced at the bottom of each tube so as to give a precise plane of contact between the fluids. The results are read after one hour at room temperature, the

Received for publication March 1, 1922.

¹ Leblanc, A.: Contribution à l'étude de l'immunité acquise, *La Cellule*, 1901, 18, p. 335. Ide: Hémolyse et antihémoglobine, *ibid.*, 1902, 20, p. 261. Demees, O.: Hémolyse et antihémoglobine, *ibid.*, 1907, 24, p. 423. This excellent work has been overlooked to a large extent. Even Bordet in his *Traité de l'Immunité*, 1920, fails to mention the results of his compatriots.

² *Wien. klin. Wchnschr.*, 1905, 18, p. 1055; *Centralbl. f. Bakteriöl.*, I. O., 1908, 39, p. 303.

³ *Centralbl. f. Bakteriöl.*, I. O., 1910, 54, p. 362. For review of literature, see Uhlenhuth, P., u. Steffenhagen, K.: *Die biol. Eiweiss-Differenzierung mittels der Präzipitat.*, *Handb. d. path. Mikroorganismen*, 1913, 3, p. 259; also Schmidt and Bennett, *J. Infect. Dis.*, 1919, 25, p. 207.

titer or strength of a given antiserum being the highest dilution of the antigen in which distinct precipitate forms under the conditions outlined.

As a rule, the serum of rabbits injected as described with erythrocytic extracts contains small quantities only of precipitins for the corresponding serum proteins, sometimes hardly any at all. Such serum may contain also agglutinin, lysin and opsonin for the corpuscles that furnish the antigenic extract. The results illustrated in table 1 were obtained with antierythrocytic serums from which all or nearly all the precipitins for serum proteins had been removed by selective absorption. For the purpose of absorption, antiserum is mixed with an equal quantity of the

TABLE 1
PRECIPITINS FOR AQUEOUS EXTRACTS OF RED CORPUSCLES

Antigens: Extracts of Red Corpuscles and Corresponding Normal Serums	Precipitin Serums Produced by Injections of Extracts of Red Corpuscles as Follows					
	Beef	Dog	Horse	Human	Sheep	Swine
Extract of beef corpuscles.....	20,000	0	0	0	0	0
Beef serum	0	0	0	0	0	0
Extract of dog corpuscles.....	0	24,000	0	0	0	0
Dog serum	0	0	0	0	0	0
Extract of goat corpuscles.....	800	0	0	0	3,200	0
Goat serum	0	0	0	0	0	0
Extract of guinea-pig corpuscles.....	0	0	0	0	0	0
Guinea-pig serum	0	0	0	0	0	0
Extract of horse corpuscles.....	2,000	0	40,000	0	0	0
Horse serum	0	0	0	0	0	0
Extract of human corpuscles.....	800	0	0	5,000	0	0
Human serum	0	0	0	32	0	0
Extract of monkey (rhesus) corpuscles...	800	0	0	3,200	..	0
Monkey serum	0	0	0	0	0	0
Extract of rabbit corpuscles.....	0	0	0	0	0	0
Rabbit serum	0	0	0	0	0	0
Extract of rat corpuscles.....	0	0	0	0	0	0
Rat serum	0	0	0	0	0	0
Extract of sheep corpuscles.....	800	0	0	0	6,400	0
Sheep serum	0	0	0	0	16	0
Extract of swine corpuscles.....	0	0	0	0	0	3,200
Swine serum	0	0	0	0	0	0

corresponding serum in a dilution of 1:200; this mixture is left at room temperature for one hour or so, placed in the icebox overnight and then centrifugated thoroughly. It should be noted that on account of this treatment the precipitin serums in table 1 are all diluted one-half. As stated, the figures in the table give the highest dilutions of extract and serums in which definite precipitate formed; this is true also of table 2; in all cases save serums the figures give really the dilutions of the whole blood because in each case the unit is the extract or hemoglobin from the corpuscles in one volume of blood. The results speak for themselves. The erythroprecipitins produced with extract of dog, horse and swine corpuscles are strictly specific, being apparently species specific

as well as cell specific; in the case of the antiserum for extract of human corpuscles a small amount of precipitin for serum persists after absorption with human serum as described, and the action of the main precipitin is strongly marked on extract of monkey corpuscles; the sheep erythroprecipitin acts almost as well on extract of goat corpuscles as on extract of sheep corpuscles, and a small amount of precipitin for sheep serum persists after absorption; the precipitins produced in response to injections of beef corpuscles act on extract of goat, horse, human, monkey, and sheep corpuscles as well as on the extract of beef corpuscles and, as one might expect, in much higher dilutions of this last extract than of any of the others. In this case the precipitins are apparently cell specific but not strictly species specific, and the corpuscles of the mammals in question seem in some degree identical. We may return to the problem of the identity of antigens in the crude aqueous extracts of these mammalian corpuscles later. We would emphasize now that evidently red corpuscles contain antigenic elements that ordinarily do not occur to any marked extent in the corresponding serum, and the question arises: What may the nature of these erythrocytic constituents be?

First the effect of passing illuminating gas and H_2S through extracts of red corpuscles was tested, and it was found that this treatment in no way interfered with the precipitation of specific antiserum. Conversion of hemoglobin into methemoglobin was also without effect.

The next step was to study the effects of splitting the hemoglobin in the extract into hematin and globin. It was quickly shown that treating erythrocytic extracts with hydrochloric acid destroyed the elements concerned in the precipitin reaction, except with highly dilute solutions of the acid, and even then there was considerable loss. With acetic acid, however, followed by ammonia, splitting of the hemoglobin with preservation of a definite specific element was accomplished. Using a 1:50 extract (on the basis of whole blood) of human corpuscles, we mixed 30 cc of extract with 20 cc of a 1/10 N acetic acid; to small quantities of this mixture increasing quantities of 1/10 N ammonia were added and enough NaCl to make a 0.9% solution. The slightly acid, clear mixtures of this kind gave precipitation with the antiserum in as high dilutions as the untreated extract and no reaction with normal rabbit serum; making the reaction alkaline and removing the voluminous precipitate of the globulin together with a part of hematin by centrifuga-

tion, still left the specific precipitinogenic elements in the solution without perceptible diminution. Near the point of neutralization there was, however, some loss of precipitinogens.

Similar results have been obtained with extracts of horse, beef, sheep and swine corpuscles. In no case did globin itself dissolved in the smallest possible amount of 1/10 N acetic acid followed by partial neutralization, or in neutral solution obtained by dialyzing, give any specific precipitate with antiserum. As yet we have not succeeded in separating the antigen absolutely from all traces of hematin as the treatment necessary with ether alcohol destroys the antigen, but the efforts are being continued. Of course, hematin itself hardly can be the antigen because it is a comparatively simple compound, probably identical in every species, and Schmidt and Bennett⁴ state that Gay on direct tests obtained no evidence of hematin being antigenic. Furthermore, in our experiments the quantity of antigen was not reduced as part of the hematin was removed with the globin precipitate.

Treating antiserum with equal quantities of the corresponding original extract or of solution, from which the globin had been removed in the manner described, diluted in either case to correspond to 1:200 of the whole blood, resulted in each instance in the complete removal of all the precipitins in the antiserum, thus indicating that the globin-free solution contained practically all the antigenic elements in the original extract.

Injecting rabbits with repeated amounts of the globin-free extracts of corpuscles resulted in the production of antisera that seem to be fully as specific in reaction as the antisera produced by injecting the original extracts, being limited in their action to globin-free derivatives of extracts and the extracts themselves.

We next turned our attention directly to the question of the relation of hemoglobin itself to the antigenic properties of extracts of red corpuscles. Horse hemoglobin recrystallized 4 times by the ammonium sulphate method reacted with the antiserum in about as high a dilution as the original extract. In this treatment most of the hemoglobin was turned into methemoglobin. The Marshall-Welker⁵ aluminum cream method is believed to remove all proteins other than hemoglobin from solution, but even after this treatment, repeated 4 times, horse, beef, sheep, swine and human hemoglobin reacted with their respective antisera just as before; and removal of the globin from hemoglobin,

⁴ Jour. Infect. Dis., 1919, 25, p. 207.

⁵ Jour. Am. Chem. Soc., 1913, 35, p. 820.

purified with aluminum cream, by means of acetic acid and ammonia still left the precipitinogenic elements in the globin-free solutions. Treatment of globin-free solutions, diluted to correspond to 1/100 of blood, with aluminum cream resulted, however, in the complete removal of the precipitinogens, which appear to be protected against the action of this cream only when hemoglobin as such is present.

TABLE 2
SPECIFIC PRECIPITINOGENIC EFFECTS OF HEMOGLOBIN AND GLOBIN-FREE SOLUTIONS

Antigens	Antiserums Produced by Injecting Rabbits								
	Beef		Horse		Human		Sheep		Swine
	Hemo- globin	Globin- free Solution	Hemo- globin	Globin- free Solution	Hemo- globin	Globin- free Solution	Hemo- globin	Globin- free Solution	Hemo- globin
Beef:									
Hemoglobin.....	50000	12800	0	0	0	0	0	0	0
Globin-free solution...	12800	12800	0	0	0	0	0	0	0
Serum.....	10	40	0	0	0	0	5	10	0
Horse:									
Hemoglobin.....	0	0	5000	4000	0	0	0	0	0
Globin-free solution...	0	0	4000	2000	0	0	0	0	0
Serum.....	0	0	0	0	0	0	0	0	0
Human:									
Hemoglobin.....	0	0	0	0	6400	6400	0	0	0
Globin-free solution...	0	0	0	0	3200	3200	0	0	0
Serum.....	0	0	0	0	320	320	0	0	0
Monkey:									
Hemoglobin.....	0	0	0	0	3200	800	0	0	0
Sheep:									
Hemoglobin.....	0	0	0	0	0	0	50000	25000	0
Globin-free solution...	0	0	0	0	0	0	25000	25000	0
Serum.....	0	0	0	0	0	0	0	0	0
Goat:									
Hemoglobin.....	0	0	0	0	0	0	25000	12800	0
Swine:									
Hemoglobin.....	0	0	0	0	0	0	0	0	50000
Globin-free solution...	0	0	0	0	0	0	0	0	12800
Serum.....	0	0	0	0	0	0	0	0	0

As 100% on Sahli's scale corresponds to 17.3 grams hemoglobin in 100 cc, the actual amount of hemoglobin in different solutions may be computed by means of the hemoglobinometer. A solution on the basis of blood 1 to 50 corresponds approximately to a solution of one part of hemoglobin in 300. Consequently, if the figures for hemoglobin in this table are multiplied by 6 the products will indicate the approximate dilution of pure hemoglobin in each case.

By injecting rabbits with the purified hemoglobins of the species mentioned and with the globin-free solutions thereof, we obtained strictly specific precipitin sera whose action is limited to the hemoglobin and globin-free solutions used as antigens in each case as shown in table 2. In case of the human antigens the antiserum formed precipitate with a low dilution of the corresponding serum due possibly to the hemoglobin in the serum which gave a positive benzidin reaction. These antisera do not contain any agglutinin, lysin or opsonin for the

corpuscles that furnish the antigenic hemoglobin, a fact of some interest in connection with the question whether various antibody effects depend on one or several distinct substances.

DISCUSSION

Hemoglobin has been the object of many investigations, but only few deal with the question whether it is responsible for the antigenic properties of extracts of red corpuscles. Demees¹ purified hemoglobin by the ammonium sulphate method and tried to determine its relation to the production of hemolysin; he obtained precipitins which he attributed to the antigenic action of hemoglobin, but no lysin, and he noted especially that a yellow precipitate developed under certain conditions. Our results appear to confirm Demees. In the more recent investigations of the antigenic properties of hemoglobin and its protein constituent, globin, diametrically opposite results have been obtained. In the course of our work certain reasons for this divergence have appeared.

First, we find that hydrochloric acid, except in very weak concentration, destroys the antigenic property of the aqueous extract of erythrocytes. Gay and Robertson⁶ concluded from complement fixation and anaphylaxis tests that globin is not antigenic, but that it may be rendered so in a compound with casein. Schmidt⁷ found that a globin-albumose compound is not antigenic. Now, as the preparations of globin used in these investigations were obtained by strong hydrochloric acid (20 cc of concentrated acid to 1 liter of solution), it is evident that the results do not exclude the possibility of unchanged globin being antigenic. On the other hand, Browning and Wilson,⁸ working with a much weaker solution of hydrochloric acid, came to the conclusion that antibodies against globin may be obtained, which, however, did not react with hemoglobin. We find that the antigen remains in solution after removing practically all the globin, but near the neutralization point the globin precipitate usually carries down a great deal of the antigen, and this may account for the results obtained by Browning and Wilson, who used partly an acid solution, partly an alkaline emulsion, the acid solution containing besides the globin the unknown antigen in solution and the alkaline emulsion a varying part of it partly dissolved and perhaps partly adsorbed to globin.

Browning and Wilson used complement fixation and not the precipitin method, because the globin precipitated all serums, but it does so only in stronger solutions, and all chances of error may be eliminated

⁶ Jour. Exper. Med., 1913, 17, p. 535.

⁷ Univ. of Calif. Public. in Pathology, 1916, 2, p. 157.

⁸ Jour. Path. Bacteriol., 1909, 14, p. 174.

by controls with normal rabbit serum. Besides the hematin present seems to protect the serum. We believe that the precipitin method, generally speaking, gives more satisfactory results than the other methods, besides being much simpler.

Schmidt and Bennett ⁴ prepared hemoglobin by repeated crystallization. The two methods they used, namely, 15% and 25% alcohol, do not permit the conclusion that hemoglobin is not antigenic, because alcohol we find is injurious to the antigen, especially in the presence of salts. These authors apparently were convinced that the negative results of their third method, namely ammonium sulphate, would not be decisive as the hemoglobin now was changed into methemoglobin, but we find that a solution in which hemoglobin has been changed into methemoglobin retains the antigenic properties of the original extract. Even after four recrystallizations followed by carbon dioxide treatment (Schmidt and Bennett's third method), horse hemoglobin (methemoglobin) reacted with antiserum as strongly as originally. It is possible that they might have obtained positive results had they injected more than 2 rabbits to produce antibodies. In any case, it would seem to be a good plan to begin by testing the different solutions with an antiserum previously prepared with the original extract.

Of other contributions to the study of the antigenic action of hemoglobin may be mentioned the work by Bradley and Sansum,⁹ in which they obtained positive specific results from anaphylactic experiments with hemoglobin, and the recent work by Higashi¹⁰ on the antigenic action of hemoglobin in which he appears to show that the precipitinogenic properties are retained by hemoglobin when eliminated in the urine. He holds that Klein's erythrocytic precipitin is a hemoglobin precipitin. Azuma¹¹ is said to have shown that hemoglobin precipitin is specific and applicable to medicolegal tests, a question that we may take up later. On account of its precipitin strength and the sharp limitation in action, antihemoglobin serum may prove of value as a test for blood in feces.

Our results appear to indicate that hemoglobin may be a specific antigen and thus they may help to throw light on its constitution. In extension of the work, the isolation of the unknown antigen and its relation to native hemoglobin are problems that demand special attention. At present we believe the following conclusions are justified:

⁹ J. Biol. Chem., 1914, 18, p. 497.

¹⁰ Jap. Med. World, 1922, 2, p. 52.

¹¹ Ibid., p. 85.

SUMMARY

Aqueous extracts of red corpuscles give rise in rabbits to precipitins the action of which appears to be limited to erythrocytic constituents, in some cases of the species furnishing the corpuscles only, in other cases extending also to such constituents of related species.

While other antigens may be present in crude aqueous extracts of corpuscles, the main precipitinogen seems to be hemoglobin, which is shown to be a species specific precipitinogen in confirmation of the early work of Ide and his pupils.

Conversion of the hemoglobin in extracts of red corpuscles into carboxyhemoglobin, sulphydrohemoglobin or methemoglobin does not affect the specific serum precipitation of the hemoglobin.

On splitting hemoglobin into hematin and globin by means of acetic acid, the precipitinogenic elements remain in the solution after removal of the globin, which does not appear to be responsible for the antigenic properties of the hemoglobin, the globin-free solution, however, being antigenic not only in tests with antiserum but also on injection in rabbits.

While the precipitinogens in extracts of red corpuscles and in hemoglobin may exist independently of hemoglobin after treatment with acids, they ordinarily are attached closely to the hemoglobin molecule, not being removed or diminished in proportion to the amount of hemoglobin by repeated crystallization or by treatment with aluminum cream, the antigen being apparently either closely adsorbed to the hemoglobin molecule or forming a part of it which can be split off by acids.

THE STREPTOCOCCI OF THE BOVINE UDDER

IV. STUDIES OF THE STREPTOCOCCI *

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The streptococci offer an interesting and important field for study because among them are found harmless and even useful organisms as well as extremely virulent ones.

Milk is perhaps the most important harbinger of the harmless streptococci, but it may at times contain pathogenic forms. While milk cannot be considered a source of any micro-organism, it is an ideal food for most forms and may therefore contain many kinds of streptococci with which it has been contaminated.

In studying the literature on the streptococci we have been impressed with the extensive researches which have been conducted in this field, but felt that it was difficult from the results to obtain a clear conception of the common types of streptococci in milk. In the older studies tests were used for purposes of classification which varied from those used in later work. Often the classification was based largely on morphology and blood-plate reactions, and in other cases largely on the fermentation of test substances. Furthermore, selective methods of isolation have added other complications.

The confusion as to the streptococci in milk has led to various interpretations as to their significance in it and in other dairy products. Their presence has been considered by some authorities as an indication of fecal contamination from the cow and by others as an indication of inflammation of the udder. In such cases the presence of the streptococci was considered the significant thing, and no attention was given as to what kind they might be. It has been felt that further studies of the streptococci, using all the most valuable physiologic tests for their differentiation, would prove valuable. For this reason the studies reported in this paper were undertaken.

Received for publication March 27, 1922.

* The preceding articles of this series were:

I. The Thermal Death Point and Limiting Hydrogen-Ion Concentration of Pathogenic Streptococci, *Jour. Infect. Dis.*, 1918, 23, p. 290.

II. The Production of Ammonia and Carbon Dioxide by Streptococci, *ibid.*, 1921, 29, p. 235.

III. Differentiation of Hemolytic Streptococci from Human and Bovine Sources by the Hydrolysis of Sodium Hippurate, *ibid.*, 1922, 30, p. 388.

In this paper we shall take up the streptococci of the udder and in later papers the streptococci from other sources as well as those found in milk at various stages of souring. No attempt has been made to review the literature completely, for this has been done quite thoroughly by other investigators.

METHOD OF ISOLATING CULTURES

Samples of milk were obtained by milking directly into sterile tubes. In every case the first two or three streams were rejected. Composite samples were taken which included each quarter of the udder.

The samples were then taken to the laboratory and plated on blood agar. The agar had the following composition: 500 cc infusion broth, 10 gm. peptone (Fairchild), 5 gm. NaCl, 500 cc distilled water, and 15 gm. shred agar. The reaction was adjusted to P_H 7.5.

This medium was sterilized in tubes in 12 cc amounts. At the time of plating the agar was melted and cooled to 45 C., and from 0.66 to 1 cc of defibrinated horse blood was added to each tube of agar before it was poured into the plates. This method is substantially the same as that described by Brown¹.

After 48 hours' incubation at 37 C., cultures were made from plates representing each sample of milk from a cow. About 10 colonies which appeared to be streptococci were fished and inoculated into infusion-broth tubes and the cultures examined microscopically after 24 hours' incubation at 37 C.

Cultures were considered streptococci when chains of four or more cells were present in sufficient numbers to show that this grouping was not accidental. After microscopic examination the cultures were replated and then transferred to agar slopes for stock.

CULTURAL METHODS

Hemolysis.—This was determined by plating on the blood agar previously described, and the results were recorded according to the methods of Brown. The plates were incubated at 37 C. and examined after 24 and 48 hours and again after 24 hours' refrigeration.

The beta type of reaction on blood plates as described by Smith and Brown² consists of a sharply defined clear zone about the colonies. Under the microscope no blood corpuscles are seen near the colony.

¹ Monograph 9, Rockefeller Institute for Medical Research.

² Jour. Med. Research, 1915, 31, p. 455.

The alpha type of Smith and Brown is quite different. After 48 hours' incubation at 37 C., the zone about the colonies of 1-2 mm. appears greenish, with a partial hemolysis or decolorization of the blood corpuscles near the colony. After refrigeration for 24 hours a clearer zone appears beyond the partly hemolyzed or decolorized zone. The corpuscles in the outer zone are decreased in number. The size of the second clearer zone varies with different cultures, and it may be seen sometimes with the naked eye. The colonies should be examined, however, with a low power lens.

The gamma type of streptococcus described by Brown is one whose colonies do not produce any perceptible hemolysis or decolorization of the blood medium during incubation or refrigeration. Among our cultures of streptococci were many which produced no change other than a greenish color in the medium about the colony, and these are designated as gamma-G types. Brown found that all of his green-producing strains could be included in the alpha type, but many of our green producers did not show sufficient indication of alpha hemolysis to permit them to be included with this type.

Methylene Blue Test.—In this test whole milk was sterilized in tubes in 10 cc amounts. A 0.1% solution of medicinal methylene blue was also prepared and sterilized, then 1 cc of this solution was added to each tube of sterile whole milk. The tubes were shaken and incubated before inoculation to insure sterility. After inoculation the reduction of the dye and the condition of the milk were recorded each 24 hours for a period of 7 days.

Fermentation Tests.—The basic medium had the following composition: 10 gm. peptone (Bacto), 10 gm. yeast (Cerevisine, French preparation), and 1000 cc distilled water. The reaction was adjusted to about P_H 7.5.

To this basic medium 1% of the various test substances was added, and the medium was then sterilized. The hydrogen-ion concentration was determined by means of indicators after 7 days' incubation at 37 C. with dextrose, lactose, saccharose, and salicin, but after 14 days with mannite, raffinose, and inulin.

*Carbon Dioxide and Ammonia Tests*³—The ability of the streptococci to produce carbon dioxide and ammonia was determined by the method described by Ayers, Rupp, and Mudge⁴. For the production

³ We are indebted to Dr. Philip Rupp of these laboratories for making the ammonia determinations and the tests for the hydrolysis of sodium hippurate.

⁴ Jour. Infect. Dis., 1921, 29, p. 235.

of CO_2 from peptone medium A was used, and medium S for CO_2 from dextrose, both in 15 cc amounts. Eldredge tubes were used for the measurement of the amount produced. The production of ammonia was determined in medium B by the Folin method. The composition of these mediums is given in the paper of the authors just quoted.

Sodium Hippurate Test.—In the determination of the hydrolysis of sodium hippurate, the cultures were grown in the peptone-hippurate medium described by Ayers and Rupp.⁵ When the organisms grew with difficulty, beef infusion broth with sodium hippurate was used. The hydrolysis of the hippurate with the formation of benzoic acid was determined in most cases by distillation, as described by Ayers and Rupp, but with some of the cultures the ferric chloride and acid test was used as described by the same authors.

CULTURAL CHARACTERISTICS OF THE STREPTOCOCCI OF THE UDDER

Most of the cultures in our collection came from the udders of apparently normal cows. A few, however, were from cows having mastitis. These cultures were not separated from the others, because we have not found any particular organism to be characteristic of inflamed udders. The types found in the normal udders were the same as in the cows with mastitis, the only difference being in the larger numbers found in cases in which mastitis was present. The 100 cultures studied were obtained from 55 samples of milk taken from 54 cows in two herds near Washington. One cow was examined twice.

Instead of recording in this paper the cultural characteristics of every culture, similar cultures have been grouped together, as is shown in table 1. It must be realized that not every culture showed the exact P_H values here given for fermentation test. There was of course a slight variation, but the figures represent the general limits reached. The range in cc of CO_2 from 15 cc of medium is given. For NH_3 the range in mg. of $\text{NH}_3\text{-N}$ per 100 cc of medium in excess of control is shown. For convenience in discussing the results, each group of cultures having the same reactions have been given one letter.

It will be observed that groups A, B, C, and D comprised 79 of the 100 cultures, and of these 64 gave the beta type of hemolysis; in other words, these cultures were hemolytic on blood plates.

⁵ Ibid., 1922, 30, p. 388.

TABLE 1
CHARACTERISTICS OF STREPTOCOCCI OF THE UDDER

Group	Num- ber of Cul- tures	Morphology	Hemol- ysis	Methylene Blue Test	Litmus Milk Reaction		P _n in Fermentation							C e of CO ₂ From		NH ₃ N Mg. per 100 C e Excess Over Control	Sodium Hip- purate Hydroly- zed	Streptococcus mastitidis		Streptococcus acidominus and varieties
					37 C.	10 C.	Dex- trose	Lac- tose	Saccha- rose	Sali- cin	Man- nite	Raffi- nose	Inu- lin	Pep- tone	Dex- trose			Var. Beta	Var. Gamma	
A	38	Chains averag- ing 60-80 cells	Beta	—	Acid, coagu- lated, $\frac{1}{2}$ de- colorized	No change	4.5 +	4.5 +	4.5 +	7.3 —	7.3 —	7.3 —	7.3 —	Range 2.92-7.57 +	—	+	+	+	+	
B	26	Chains averag- ing 60-80 cells	Beta	—	Acid, coagu- lated, $\frac{1}{2}$ de- colorized	No change	4.5 +	4.5 +	4.5 +	7.3 +	7.3 —	7.3 —	7.3 —	3.25-5.91 +	—	+	+	+	+	
C	7	Chains averag- ing 60-80 cells	Gamma, some green	—	Acid, coagu- lated, $\frac{1}{2}$ de- colorized	No change	4.5 +	4.5 +	4.5 +	7.2 —	7.2 —	7.2 —	7.2 —	4.08-6.50 +	—	+	+	+	+	
D	8	Chains averag- ing 60-80 cells	Gamma, some green	—	Acid, coagu- lated, $\frac{1}{2}$ de- colorized	No change	4.5 +	4.5 +	4.5 +	7.2 —	7.2 —	7.2 —	7.2 —	4.57-5.78 +	—	+	+	+	+	
E	3	Chains of 4-10 cells	Gamma, some green	+	Acid	Slight acid	4.6 +	4.5 +	4.6 +	4.6 +	4.6 +	7.4 —	4.6 +	5.20-6.99 +	—	+	+	+	+	
F	2	Chains of 4-40 cells	Alpha weak	+	Acid	Slight acid	4.5 +	4.5 +	4.5 —	4.5 +	4.5 +	7.3 —	7.4 —	4.57-5.78 +	—	+	+	+	+	
G	4	Chains of 4-20 cells	Alpha weak	May decoh- orize after 5 days	Acid, may be slightly coagulated	No change	4.5 +	4.8 +	4.5 +	7.4 —	7.3 —	4.6 +	7.4 —	2.86-4.09 +	—	±	±	—	—	
	4	Chains of 4-10 cells, sometimes longer	Alpha	—	No change	No change	6.2 +	6.2 +	6.2 +	7.3 —	7.3 —	7.5 —	7.5 —	2.60-3.18 +	—	+	+	+	+	
	6	Chains of 4-10 cells, sometimes longer	Alpha	—	No change ²	No change	6.2 +	6.2 +	6.2 +	6.0 +	7.3 —	7.5 —	7.5 —	3.25-10.37 +	—	+	+	+	+	
	1	Long chains of hundreds of cells	Alpha	—	No change	No change	6.3 +	6.4 +	6.1 +	6.1 +	7.4 —	7.5 —	6.2 +	5.98 +	—	+	+	+	+	
	1	Chains of 20-40 cells	Gamma	—	No change	No change	6.2 +	6.4 +	6.4 +	6.4 +	6.3 +	7.3 —	7.4 —	4.16 +	—	+	+	+	+	

* Note that no NH₃ is formed.

The 79 cultures comprising the first 4 groups were all long-chain-forming streptococci. Groups A and B, 64 cultures, were hemolytic; while C and D were not, but were of the gamma or gamma-G type. The beta types varied in the fermentation of salicin, and the same may be said of the gamma types. The cultures of all 4 groups formed CO_2 and NH_3 from peptone, but no CO_2 from dextrose. Particular attention is called to the fact that they all hydrolyzed sodium hippurate, forming benzoic acid and glycollol.

Because of our nonselective method of isolation, we feel that these streptococci represent the "majority streptococcus flora" of the cow's udder. They may be grouped and called *Streptococcus mastitidis* (Guillebeau). There are apparently two varieties, hemolytic and non-hemolytic, which may be termed beta and gamma varieties.

Nocard and Mollereau⁶ in 1887 isolated a long-chain-forming streptococcus from a case of contagious mastitis; and from the rather meager description it would appear to be the common udder type encountered in our studies. It is interesting to observe that Nocard and Mollereau found that they could reproduce the disease by inoculation. The organism appears to have been termed *Streptococcus mastitidis contagiosae* by Guillebeau.⁷ Jensen⁸ has also applied the name streptococcus mastitidis to the organism causing mastitis.

STREPTOCOCCUS MASTITIDIS

Streptococcus mastitidis is characterized as follows: It does not reduce methylene blue; coagulates litmus milk usually in 24 hours, and partially decolorizes the milk after coagulation; does not grow in milk at 10 C. It ferments dextrose, lactose, and cane and may or may not ferment salicin. It does not ferment mannite, raffinose and inulin. It produces CO_2 and NH_3 from peptone, but no CO_2 from dextrose. It hydrolyzes sodium hippurate into benzoic acid and glycollol. There are two varieties; one which produces the beta type of reaction, and the other which produces the gamma type on blood-agar plates.

Streptococcus mastitidis seems to agree in the ordinary cultural characteristics with the *Streptococcus pyogenes* type found to be common in the udder by Rogers and Dahlberg.⁹ Sherman and Albus¹⁰ also found the streptococcus pyogenes type, fermenting dextrose, lactose,

⁶ Ann. Inst. Pasteur, 1887, 1, p. 109.

⁷ Landw. Jahrb. Schweiz, 1890, 4, p. 27.

⁸ Mem. d. l'Acad. Roy. d. sc. et Lettr. de Danemark, Sect. d. Sc., 1919, 5.

⁹ U. S. Dept. Agr. Jour. Agr. Research, 1914, 1, p. 491.

¹⁰ Jour. Bacteriol., 1918, 3, p. 153.

saccharose, and sometimes salicin, to be the characteristic streptococcus of the udder; and they pointed out the value of the negative reduction of methylene blue and the inability of this type to grow at 10 C. Jones¹¹ in a study of streptococci from the udder of cows having mastitis, found that they could be separated into 2 groups, one fermenting dextrose, lactose, maltose and saccharose, and the other salicin in addition. Both his hemolytic and nonhemolytic cultures fell in these 2 groups.

The "typical udder type," as we may term *Streptococcus mastitidis*, might be given various names according to various classifications. For example, by Holman's¹² scheme, group A could be called *St. anginosus*; group B, *St. pyogenes*; group C, *St. salivarius*; and group D, *St. mitis*.

Classification on this basis would, in our opinion, be incorrect, because it is based only on the fermentation of a few sugars and similar substances and hemolysis.

In view of the results obtained by the use of tests devised in these laboratories, such as the difference in P_H , the production of CO_2 and NH_3 from peptone, and the hydrolysis of sodium hippurate, we do not feel certain that the typical udder type is commonly distributed; nor are we sure that it is limited to the udder. This cannot be known until the differential tests are extensively applied to large numbers of streptococci from varied sources. We do believe that the typical udder type is not *Streptococcus pyogenes*.

According to Holman's classification, group B in table 1 would be called *Streptococcus pyogenes*, and so would the majority of human hemolytic types in our collection. In table 2 will be seen the principal cultural characteristics of 33 human hemolytic streptococci. Most of these were isolated from pathologic sources, with a few from normal throats.

On the basis of hemolysis and positive or negative fermentations the first 23 cultures would be identical with group B of the udder, but the use of other tests show that they are quite different.

Although both human and bovine types show the beta type of hemolysis and appear quite similar on blood plates, the human type is about 100 times more hemolytic than the bovine type, as shown by the hemolysis in tubes. This point has also been brought out by Brown.¹³ In

¹¹ Jour. Exper. Med., 1918, 28, p. 149; *ibid.*, p. 253.

¹² Jour. Med. Research, 1916, 34, p. 377.

¹³ Jour. Exper. Med., 1920, 31, p. 35.

the methylene blue test and milk reaction there is little difference, although the human types are not quite so active in producing acidity in milk.

The P_H reached in the fermentation of test substances is distinctly different. The udder types reach about 4.5, while the human type produces less acid and reaches about P_H 5.5. This reaction was obtained in a dextrose yeast (Cerevisine) peptone broth. The final P_H can be varied, as has been shown by several investigators, but it is quite constant when the test is carried out in a definite medium.

Horry Jones¹⁴ has shown that the hydrogen-ion concentration of human types of hemolytic streptococci can be increased; and F. S. Jones¹⁵ has also shown that the so-called P_H limits can be changed by variation of the medium.

TABLE 2
CULTURAL CHARACTERISTICS OF HEMOLYTIC STREPTOCOCCI FROM HUMAN SOURCES, MOSTLY FROM PATHOLOGIC CONDITIONS

No. of Cultures	Hemolysis	P_H in Fermentations							CO ₂ from		NH ₃ from Peptone	Sodium Hippurate Hydrolyzed	Holman's Classification
		Dextrose	Lactose	Saccharose	Salicin	Man-nite	Raffinose	Inulin	Pep-tone	Dextrose			
23	Beta	5.4 +	5.4 +	5.4 +	5.5 +	7.4 —	7.4 —	7.4 —	+	—	+	—	Streptococcus pyogenes
9	Beta	5.4 +	5.4 +	5.4 +	5.5 +	5.5 +	7.4 —	7.4 —	+	—	+	—	Streptococcus infrequens
1	Beta	4.6 +	4.6 +	4.6 +	4.6 +	7.2 —	4.5 +	7.1 —	+	—	+	—	

"The limiting P_H " has been the term commonly used, and it is defined by Jones¹⁵ as the acid production in a given medium which limits the final growth. We believe that the term "final P_H " is less misleading than "the limiting P_H ." The final P_H of a culture is not necessarily the limiting P_H , and we feel that the final P_H is that reached when growth ceases. It may be the limiting P_H in some cases, or it may be the result of double fermentations with a reversion of reaction as in the case of *B. aerogenes*. One is inclined to think of a limiting P_H , or the acid tolerance of an organism, as being quite a definite thing and not subject to much variation; while on the other hand, it is readily seen that the final P_H could be subject to variation due to the composition of the medium for growth.

¹⁴ Jour. Infect. Dis., 1920, 26, p. 160.

¹⁵ Jour. Exper. Med., 1920, 32, p. 273.

The final P_H of the streptococci and the difference between human and bovine types of hemolytic organisms have been discussed somewhat extensively, because we feel that this difference is quite fundamental and not of accidental occurrence.

Proceeding with the comparison of group B of the udder type and the 23 cultures of human type, it will be seen from tables 2 and 3 that both produce CO_2 and NH_3 from peptone and no CO_2 from dextrose. A further distinction between the two types is the negative hydrolysis of sodium hippurate by the human type and the positive reaction by the udder type. This test has been described by Ayers and Rupp.⁵

These differences in final P_H and in the hydrolysis of sodium hippurate hold for all udder types and human types, with the exception of one culture from a normal human throat. This culture reached a final P_H of about 4.6 but did not hydrolyze the hippurate. It also fermented raffinose. This type may be common in normal throats, but we have no information on this point, except that, as is generally known, raffinose fermenters are common in saliva.

CHARACTERISTICS OF STREPTOCOCCUS MASTITIDIS AND STREPTOCOCCUS PYOGENES

Streptococcus mastitidis, Var. Beta Var. Gamma		Streptococcus pyogenes
Beta type (weak)	Blood-agar Plate.....	Beta type (strong)
Gamma type	Dextrose.....	* P_H about 5.5 in dextrose-yeast broth
* P_H about 4.5 in dextrose-yeast broth	Lactose.....	+
+	Saccharose.....	+
(±)	Salicin.....	(±)
—	Mannite.....	—
—	Raffinose.....	—
—	Inulin.....	—
+	CO_2 from peptone.....	+
—	CO_2 from dextrose.....	—
+	Sodium hippurate hydrolyzed.....	—*
Coagulated, partly decolorized after coagulation	Milk at 37 C.....	Acid, may be coagulated
No growth	Milk at 10 C.....	No growth
No decolorization	Methylene blue, milk.....	No decolorization

* Note particularly these reactions.

These differences between the streptococci of the udder and hemolytic streptococci from human sources have been discussed at length in order to show why we believe they are not the same species.

For the sake of clearness, we have listed the characteristics of what we consider *Streptococcus mastitidis* and also *Streptococcus pyogenes*.

Returning to table 1 again, it will be seen that groups E and F are not included in the *Streptococcus mastitidis* group. One group shows

the alpha type of hemolysis, and both reduce methylene blue, they form acid in milk, and grow at 10 C.; both ferment mannite, and one group ferments inulin. In the rest of their characteristics they agree with *Streptococcus mastitidis*.

Group G is distinctly different in that the organisms produce CO₂ in infusion peptone broth without NH₃. There were some indications that the CO₂ came from organic acid salts.

The 12 remaining cultures represent what appears to be a new species of streptococcus, which has been named merely as a matter of record. They generally showed the alpha type of hemolysis, which in most cases was very distinct. No change was observed in litmus milk, and they produced little acid in fermentation tests. This is the most characteristic feature of these organisms. In the yeast-peptone medium the P_H decreased from 7.5 to about 6.2. This medium was lightly buffered, and for this reason the weak fermentations were observed. There was considerable variation in the test substances fermented. These organisms also formed CO₂ from infusion-peptone broth; but no NH₃. Here again the CO₂ seemed to come from organic acid salts. Because of their ability to produce only a little acid from test substances, we have termed these cultures varieties of *Streptococcus acidominimus*. These organisms apparently are different from those observed by Holman¹² and called *Streptococcus ignavius* on account of their lack of fermentative ability.

STREPTOCOCCUS ACIDOMINIMUS

Streptococcus acidominimus is characterized by the small amount of acidity developed in the fermentation of test substances. The change in P_H is about 1.0- 1.5, say from 7.5 to 6.5 in a lightly buffered medium. Hemolysis is usually of the alpha type. No change is noted in the litmus milk. CO₂ is produced from a 4% peptone-infusion broth medium, but not from dextrose. No ammonia is formed as is usually the case when CO₂ is formed. The CO₂ probably comes from organic acid salts. Sodium hippurate is hydrolyzed to benzoic acid and glycollol. There are several varieties of *Streptococcus acidominimus* which differ in the test substances fermented.

FREQUENCY OF THE PRESENCE OF STREPTOCOCCI IN THE UDDERS OF COWS

Streptococci occur frequently in the udders of normal cows. This has been shown by Sherman and Hastings,¹⁶ Evans,¹⁷ and also by

¹⁶ Creamery and Milk Plant Monthly, 1915, 3, p. 11.

¹⁷ Jour. Infect. Dis., 1916, 18, p. 437.

Jones.¹⁸ In our studies streptococci have been isolated by direct platings of milk directly from the udder of 51 of the 133 normal cows examined. This represents about 38% of the animals tested. They were isolated from each of 17 cows having mastitis, and varied in 3 cases, from a few thousand to many millions. In some animals with mastitis the predominating streptococcus was the hemolytic (beta) variety and in other cases the nonhemolytic (gamma).

It seems evident that *Streptococcus mastitidis* is commonly found in the udders of normal cows, and also to a less extent other species of streptococcus. The fact that milk containing these organisms has been consumed regularly with no ill effects indicates that these streptococci need not be feared. In this connection the experiments of Nocard and Mollereau^{6, 11} are of interest. They isolated a streptococcus, apparently *Streptococcus mastitidis*, and fed cultures to dogs and rabbits. No ill effects were noted, and they concluded that milk containing these organisms could be used for food without danger. Jones¹¹ also fed nonhemolytic udder streptococci to a pig, with no bad results.

SUMMARY AND CONCLUSIONS

1. The typical streptococcus of the udder of the cow was found to be *Streptococcus mastitidis*. Cultural characteristics of two varieties of this organism are described.

2. *Streptococcus mastitidis* is practically identical with *Streptococcus pyogenes* when the usual cultural characteristics are studied. They are separated largely on the difference in final P_H and difference in ability to hydrolyze sodium hippurate.

3. Cultural characteristics are presented of a few other streptococci which are not included with *Streptococcus mastitidis*.

4. An apparently new species is described which because of the small amount of acid produced in test substances is termed *Streptococcus acidominimus*.

5. It is shown that streptococci are frequently found in the udders of normal cows and that the same species are also present in cases of mastitis. There appears to be no reason to believe that *Streptococcus mastitidis* is pathogenic for man when consumed in milk, and it can apparently be readily distinguished from *Streptococcus pyogenes*.

¹⁸ Jour. Exper. Med., 1918, 28, p. 735.

IMMUNITY IN EXPERIMENTAL PNEUMONIA

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Clinical observations have shown that an attack of pneumonia is not, as a rule, followed by lasting immunity. Rather, the contrary is true. Osler¹ says: "No other acute disease recurs in the same individual with such frequency. Instances are on record of individuals who have had 10 or more attacks. The percentage of recurrences has been placed as high as 50. Netter gives it as 31% and he has collected the statistics of eleven observers who place the percentage at 26.8%." In the great majority of cases of pneumonia, the etiologic agent is a gram-positive coccus of one species or another. It is well known that members of this group of organisms, irrespective of their mode of entrance or the location of the lesion in the body, do not ordinarily set up a reaction on the part of the cells which results in producing a persistent marked increase in resistance. Indeed, Bunting² emphasizes that it is only in those diseases that show a definite reaction on the part of the lymphoid tissue that we have any marked and lasting immunity. The cocci, on the contrary, quite generally stimulate the bone marrow resulting in the production of a polymorphonuclear leukocytosis.

It has been suggested by Cecil and Blake,³ as a result of their vaccination experiments, that it is possible that the immunity following recovery from a pneumococcus pneumonia is highly specific, and that recurring attacks may be due to one of the other fixed types not found in the initial attack or to some members of the irregular type 4.

A considerable group of workers (notably Friedberger⁴ and his collaborators, and Vaughan and Wheeler⁵) hold, on the contrary, quite the opposite opinion. They maintain that the specificity of bacteria has been overemphasized and that the important points in the production of a given clinical picture are not the particular species or strain of organ-

Received for publication March 29, 1922.

¹ Principles and Practice of Medicine, 1913, p. 76.

² Personal communication.

³ Jour. Exper. Med., 1920, 31, p. 685.

⁴ Ztschr. f. Immunitätsf., 1911, 10, p. 453.

⁵ Protein Split Products, 1913.

ism but the general location of the lesions, the invasive properties of the bacteria, and special local accumulations of the organisms.

Are the frequent recurrences of pneumonia, then, due to the nature of the organism causing the disease or to some property peculiar to the lung tissue? Is it impossible for infectious processes in the lungs to stimulate the production of any considerable amount of antibodies? Since it is feasible to produce pneumonia experimentally by the use of certain gram-negative organisms which commonly induce a persistent immunity of marked degree, it has seemed possible to answer these questions. We have so little knowledge and control of the spread of respiratory infections that such answers ought to be of some value.

B. typhosus was chosen for the work planned, because it is a gram-negative organism which stimulates a high degree of immunity and is capable of exciting a pneumonic process when it is lodged in the lung. It is also markedly different from the gram-positive cocci in that it ordinarily attacks lymphoid tissue and stimulates that tissue to the production of a lymphocytosis. It was proposed to produce pneumonia by means of *B. typhosus*, to determine whether antibody production could thereby be stimulated and if so, to compare the agglutinin titer of the blood serum from these animals with that of animals injected intravenously.

A stock culture of *B. typhosus* was used. As it was avirulent for rabbits it was necessary to raise its virulence by animal passage. Attempts to do this encountered little success so long as the organisms were recovered and grown on ordinary agar. The use of Loeffler's blood serum for transplants proved more satisfactory. The same parent strain was used for the initial injection. Following the first injection, however, the organisms recovered from animals injected by each route were kept separate and reinjected always by the same path. The intravenous strain was passed through 21 rabbits, the virulence being finally raised so that one culture proved a lethal dose.

The virulence of the intratracheal strain was raised by passage through the lungs of a series of rabbits until the injection of 12 slant cultures would produce a fatal bronchopneumonia. The method of injection was as follows: After shaving the neck and disinfecting the skin, the rabbit was anesthetized and when completely relaxed was untied and the head bent back over the end of the board so as to bring the trachea up immediately under the skin. A blunt pointed needle was inserted first through the skin and then into the trachea. After

the suspension was injected, the syringe was removed from the needle and the barrel filled with air which was then used to force the suspension well down into the alveoli. It was found that about 4 c c of fluid was all that could be safely injected into an ordinary sized rabbit by this method.

After injection in this manner, the rabbits crouched quietly in the cages and refused to eat. On the second day the breathing became labored and rapid and the animals were apparently ill. The symptoms reached their height on the third day. In the nonfatal cases recovery was rapid after this time. When death occurred before the third day the intestinal lesions were more marked than those in the lungs, with marked injection of the intestinal vessels and congestion of the lymphoid follicles. This distribution of the lesions, together with the fact that positive blood cultures were always obtained in these cases throughout the course of the disease, would indicate the early invasion of the blood stream in such acutely fatal cases. In animals that died later, the lesions in the lungs were more marked than those in the intestines, even when the blood cultures were positive at necropsy. The invasion of the blood stream in these cases took place only a short time before death, as the blood cultures up to that time were uniformly negative. The lungs in all of these intratracheal animals showed a well marked bronchopneumonia varying somewhat in the amount of lung tissue involved. Microscopically, the pneumonic lesions showed a considerable percentage of mononuclear cells in the exudate with relatively little fibrin and only slight evidence of hemorrhage. The interstitial tissues were heavily involved in the inflammatory process.

After the preliminary work was finished, 2 groups of rabbits were injected as indicated, one intravenously and the other intratracheally. Four cultures were used for intratracheal injections, a dose which was less than half the M L D determined in the preliminary titration. Of the 10 rabbits injected, however, 3 died on the second day, 1 on the third and 1 on the fourth, all with early pneumonic lesions and positive blood cultures. (It was thought that the excessively hot weather, during which these experiments were in progress, may have been responsible for an abrupt drop in the resistance of the animals although a sudden increase in the virulence of the bacteria may also have been a factor). The other 5 animals developed a definite pneumonia with typical symptoms and areas of consolidation but gradually recovered. Aside from the normal sample of blood taken before the injection, bleed-

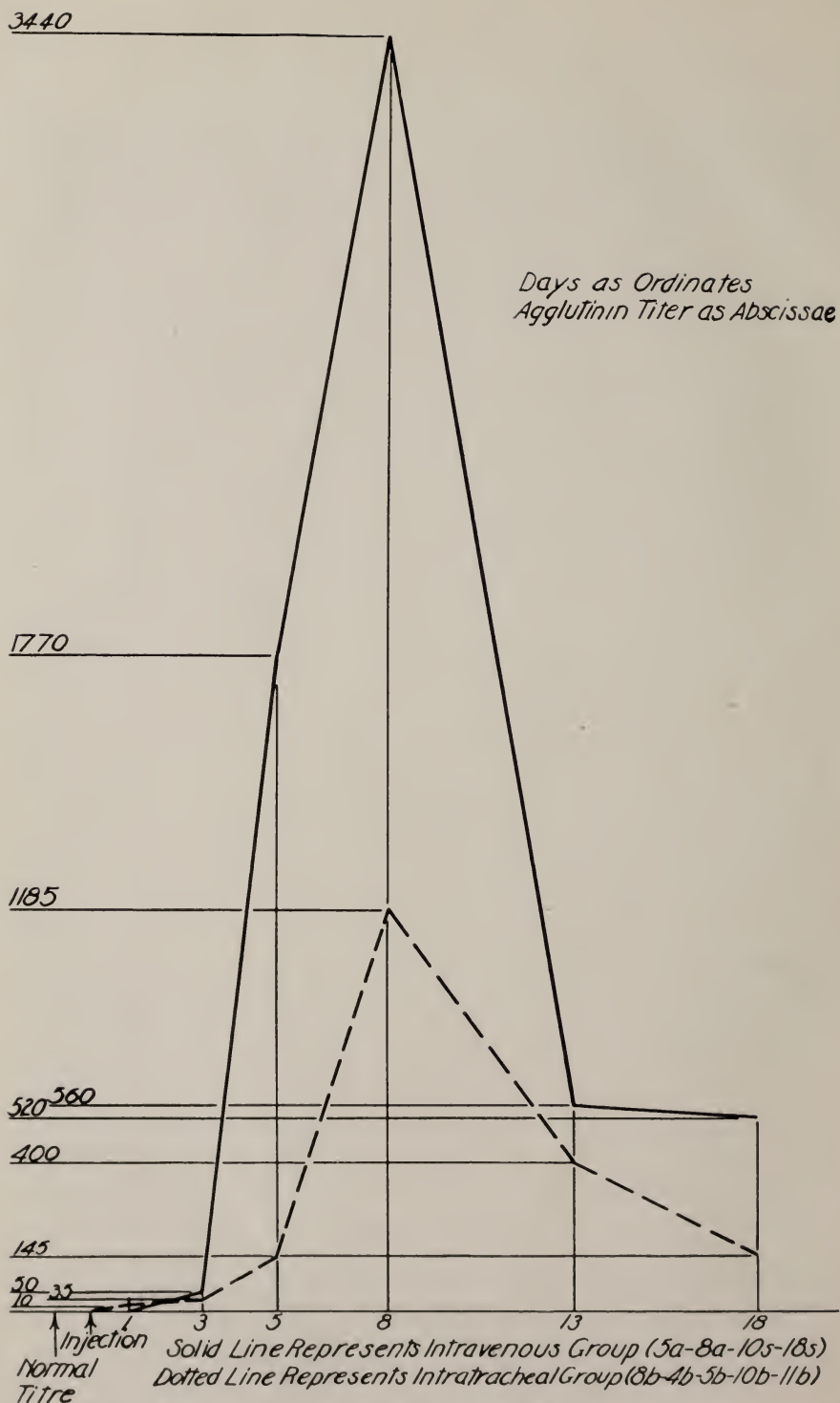


Chart 1.—Composite agglutination curves of intratracheal and intravenous groups.

TABLE 1
AGGLUTININ TITERS

Bleedings	Series A—Intravenous Injections				
	Rabbit 4a	Rabbit 5a	Rabbit 8a	Rabbit 10a	Rabbit 18a
Before injection.....	20—	20—	20—	20—	20—
Days after Injection:					
1.....	20±	20—	20±	20—	20—
3.....	20±	40+	80+	40+	40+
5.....	40+	1280+	5120+	360+	3.0+
8.....	40+	2560+	10240+	320+	640+
13.....	20+	1280+	640+	160+	160+
18.....	160+	1280+	640+	80+	80+

Bleedings	Series B—Intratracheal Injections				
	Rabbit 3b	Rabbit 4b	Rabbit 5b	Rabbit 10b	Rabbit 11b
Before injection.....	20—	20—	20±	20—	20+
Days after Injection:					
1.....	20±	20±	20±	20—	40+
3.....	20±	80+	20—	20+	80+
5.....	160+	160+	40+	40+	3.0+
8.....	320+	160+	160+	160+	5120+
13.....	160+	320+	160+	80+	1280+
18.....	160+	20+	No test	80+	320+

+ indicates definite macroscopic agglutination.

± indicates an appearance slightly more granular than control; a doubtful positive.

— indicates a completely negative reaction.

TABLE 2
BLOOD COUNTS AFTER INTRAVENOUS INJECTIONS (RABBITS 4A AND 5A)

Before and Days After Injection	Red Cor-puscles		Leuko-cytes		Poly-mor-phonuc-lears		Large Lym-pho-cytes		Small Lym-pho-cytes		Large Mono-nuc-lears		Transi-tional		Eosin-ophils		Baso-phils	
	4a	5a	4a	5a	4a	5a	4a	5a	4a	5a	4a	5a	4a	5a	4a	5a	4a	5a
Before After	5,350,000	5,430,000	10,200	9,000	41	37	7	13	49	44	3	6
1 day	5,480,000	4,830,000	13,800	9,250	70	78	4	2	23	17	1	1	2	2
3 days	4,280,000	4,810,000	24,000	14,200	73	69	13	7	8	20	6	4
5 days	3,810,000	3,850,000	8,700	10,350	57	39	21	18	19	41	1	3	1
8 days	4,050,000	3,960,000	13,100	7,900	61	46	15	18	22	30	2	6
13 days	4,460,000	3,730,000	11,300	7,950	50	31	26	20	19	42	1	..	1	1	3	6
18 days	4,470,000	4,710,000	9,000	10,600	48	50	20	18	28	26	2	2	6

TABLE 3
BLOOD COUNTS AFTER INTRATRACHEAL INJECTIONS (RABBITS 10B AND 11B)

Before and Days After Injection	Red Cor-puscles		Leuko-cytes		Poly-mor-phonuc-lears		Large Lym-pho-cytes		Small Lym-pho-cytes		Large Mono-nuc-lears		Transi-tional		Eosin-ophils		Baso-phils	
	10b	11b	10b	11b	10b	11b	10b	11b	10b	11b	10b	11b	10b	11b	10b	11b	10b	11b
Before After	4,690,000	5,170,000	11,950	8,100	42	22	22	19	33	57	1	2	2
1 day	4,880,000	5,120,000	10,300	7,650	36	68	6	12	57	20	1	1	
3 days	4,960,000	5,970,000	12,400	11,600	43	43	9	27	46	30	1	1	
5 days	5,300,000	4,210,000	8,750	10,950	57	37	22	23	20	40	1	1	
8 days	5,610,000	3,500,000	9,400	12,000	37	50	17	33	45	14	1	1	2
13 days	4,840,000	4,780,000	9,200	10,400	42	45	18	28	40	25	1	1
18 days	6,250,000	4,260,000	12,250	11,550	25	56	14	15	60	28	1	1	

ings were made from these animals 1, 3, 5, 8, 13 and 18 days after the injection. At each bleeding blood cultures were made in broth and on litmus lactose agar. Complete blood counts were also obtained from 2 of these rabbits at each of the bleedings.

The intravenous group, consisting of 9 animals, were injected with $\frac{1}{8}$ of a culture; the M L D previously determined was one culture. This group also showed a depression in resistance as 2 animals died within 24 hours with positive blood cultures at necropsy. Two others died of intercurrent infections on the 4th and 8th days, respectively. The 5 surviving animals were bled on the same days as the intratracheal group. Blood cultures were taken in each instance and complete blood counts in the case of 2 of these animals.

In making blood counts during the preliminary work a wide variation was observed in the number of both red and white corpuscles, depending on the manner in which the blood was obtained. If the ear vein was pricked and the pipet filled from the drop that escaped, the white count averaged several thousand and the red several million higher than in blood taken after the bleeding had continued for a few minutes. This observation was confirmed by repeated duplicate counts, and also by comparison of counts taken at the close of the bleeding with those of another worker who obtained the blood in the ordinary manner. For this work the blood for all counts was taken after the blood for serum had been collected.

All the serum obtained was preserved in sealed tubes in the refrigerator until the close of the experiment when it was titrated for agglutinins by the macroscopic method. The tubes were incubated one hour, refrigerated over night and read the next morning.

Tabulated results obtained with each animal and composite graphs of each group are given in tables 1, 2 and 3 and chart 1.

DISCUSSION .

The titration of the serum for agglutinins gave some interesting facts. In both groups, intratracheal as well as intravenous, the highest antibody production noted occurred on the eighth day following the injection of *B. typhosus*. The intravenous group, however, attained on the average a higher level (1: 3,440) than did the intratracheal group (1:1,185). Rabbit 11 b, however, had a normal titer of 1:20 and reached a point much higher than any of the others in the intratracheal group. Rabbit 4a of the intravenous group showed a low titer—between 1: 40 and 1: 80. Its total white and polymorphonuclear

leukocyte counts indicate that there was probably another infection which interfered with the usual antibody response. One rabbit, 6b, injected intratracheally, showed no production of antibodies. These two animals therefore (4a and 6b) were not included in making the composite curves.

Blood cultures were uniformly negative in all of the nonfatal cases. This would indicate that there were few, if any, organisms circulating in the blood stream even on the first day after injection. Those injected intravenously must have been taken up by the tissues, while those injected into the lungs either remained localized or entered the blood stream so slowly as to be picked up immediately by the spleen, liver or other tissues,⁶ so that they were never found in the blood cultures. Nevertheless, it is evident that the lung epithelium does not prevent the passage of *B. typhosus* given in large doses. The immediate deaths following intratracheal inoculation were shown by blood cultures to be due to septicemia. When death occurred after several days, however, necropsy showed that it was due to pneumonia.

The blood counts made were too few to be the basis of any definite conclusions, but are suggestive. The total white count reached its peak on the day when the clinical symptoms were most marked, and it reached a much higher level in the animals injected intravenously than in those injected by the intratracheal route. The lymphocyte count in the animals of both series showed a marked fall on the day following the injection. In the intratracheal series, this was followed by a sharp rise on the third day and a fall again on the fifth, after which, there was a gradual rise to normal on the part of one animal, and to a point above normal on the part of the other. This second animal showed a fall in polymorphonuclear leukocytes but a rise in total white cells on the eighteenth day. In the intravenous series, the lymphocyte curve reached its lowest point on the first day after injection and showed a return to normal by the fifth day. The relative proportion of large and small lymphocytes showed a marked stimulation of the lymphoid tissues following the initial destruction and injury. At the time of greatest antibody content, the blood counts had returned practically to normal and the animals were apparently well.

Although it is obvious that a larger series of animals would give a more adequate basis for conclusions, it is evident that considerable antibody production can be induced by *B. typhosus* when that organism

⁶ Bull. Jour. Exper. Med., 1915, 22, p. 475. Drinker: Ibid., 1921, 33, p. 77.

is used to produce a pulmonary lesion in rabbits. As we should expect, the absorption of the antigen is less and the antibody response is less than when, by means of intravenous injection, there is widely scattered opportunity for absorption of the antigen. The relatively greater resistance of the body when subjected to attack through the lung tissue has again been demonstrated in that by the intratracheal route the animals withstood many fatal doses as measured by intravenous tolerance. Possibly this is due partly to the fact that most of the organisms do not pass through the alveolar wall into the blood stream, and also to the failure of the alveolar epithelium to anchor physiologically much of the antigen.

A study of the bone marrow in connection with the blood counts should offer further information of interest, and a comparison of antibodies resulting from localized lesions in other parts of the body should be undertaken.

CONCLUSIONS

The injection of *B. typhosus* by the intratracheal route causes bronchopneumonia, and coincidentally with recovery specific agglutinins are found in the blood.

The agglutinin titer in the group animals with pneumonia does not reach as high a level as in the group of intravenously treated animals but the peak of the curve is reached on the same day, the eighth following the injection of the bacteria.

The failure of attacks of pneumonia to produce any considerable immunity is probably due, therefore, to the nature of the organism causing the disease rather than to any peculiar properties inherent in the lung tissue.

THE PRECIPITATION OF COLLOIDAL GOLD IN THE CEREBROSPINAL FLUID OF HORSES WITH DOURINE

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Dourine is a trypanosomiasis which, under natural conditions, affects only the horse and the ass.¹ The cause, *Trypanosoma equipedum*, is transmitted solely by coition, and two distinct stages are noted, the local lesions on the genital organs followed by an affection of the nervous system, indicated usually by a paralysis of the posterior extremities, although occasionally the forelegs and the face are affected.

Dourine was first recognized in the United States in 1886, and in spite of vigorous control measures, it continued to make its appearance from time to time in different parts of the country. Until 1912, the diagnosis of the disease rested solely on physical examination which, due to the nature of the malady, was unsatisfactory as the disease would be detected only in animals showing clinical symptoms at the time of examination. At this time Mohler, Eichhorn and Buck² perfected the application of the complement-fixation reaction to the diagnosis of dourine, and by its means the extent of the disease was definitely determined.

An active campaign was then begun, by the Bureau of Animal Industry and the various states in which dourine existed, for the control and eradication of the disease, the complement-fixation test being adopted as the official diagnostic agent.

Samples of blood serum from all breeding animals in territory in which dourine exists, or is suspected, are forwarded to the pathologic laboratory of the Bureau of Animal Industry where they are subjected to the complement-fixation test. The animals are held in virtual quarantine until the results of the test are received, and only those animals

Received for publication March 29, 1922.

¹ Mohler and Schoening: Dourine of Horse, Farmers' Bull. 1146, U. S. Dept. of Agric.

² Proceedings Am. Vet. Assn., 1913.

whose serums give a negative reaction are released for breeding. Animals whose serums give a positive reaction are destroyed and their owners reimbursed.

The greater part of the work is done in the spring and early summer and large numbers of samples are tested daily—as high as 1,800 samples have been tested in one day. Special apparatus³ designed to facilitate this work are factors in making possible the testing of large numbers of specimens with a minimum of labor and no confusion.

In view of the fact that the colloidal gold test devised by Lange,⁴ has been used with a large degree of success in classifying affections of the central nervous system, and the further fact that dourine presents, in certain stages of the disease, symptoms of paralysis as a result of degeneration of the peripheral nerves and the intervertebral ganglions, it was thought that this test might give results of scientific interest, if not practical value, when applied to the spinal fluid of infected horses.

Accordingly, we concluded to select certain animals, the blood serum of which reacted to the complement-fixation test for dourine, and obtain from them specimens of spinal fluid for examination. The specimens were collected immediately after killing the animals.

The 33 specimens of spinal fluid which were suitable for examination were subjected to the complement-fixation test for dourine, the fluids being tested in 3 amounts, 0.1, 0.25, and 0.5 cc, with an antigen⁵ composed of pure trypanosomes preserved in glycerol. Globulin tests were made according to the Ross-Jones method. The remaining fluid was then centrifuged, the lower stratum turned out on a slide, fixed and stained according to Wright's method, and examined for trypanosomes, but in no case were any found. Owing to the disintegration of cells, no reliability could be placed in cell counts. The limited amount of spinal fluid did not permit an examination for protein content. Table 1 gives the results.

In noting the results of the colloidal gold test, five reactions were recognized:

5. Supernatant fluid colorless; precipitation of gold complete
4. Pale blue
3. Blue
2. Purple or lilac
1. Red blue
0. No change

³ Buck, J. M.: Jour. Infect. Dis., 1916, 19, p. 267. Reynolds, F. H.: Jour. Agric. Research, 1918.

⁴ Berl. klin. Wehnschr., 1912, 49, p. 897; Ztschr. f. Chemotherapie, 1912, 1, p. 44.

⁵ Reynolds, F. H., and Schoening, H. W.: Jour. Agric. Research, 1918.

TABLE 1
COLLOIDAL GOLD TEST OF CEREBROSPINAL FLUID OF HORSES WITH DOURINE

Groups	Nos.	Globulin Test	Complement Fixation		Clinical Symptoms and Postmortem Findings	Colloidal Gold Test of Cerebrospinal Fluid
			Serum	Spinal Fluid		
Horses showing clinical evidence of Dourine—7	1	Neg.	0.07 c c pos.	0.1 c c \pm 0.25 c c 4+	Scars on vulva	0012321100
	2	Pos.	0.03 c c pos.	Neg.	Scars on vulva, slight posterior paralysis	0050000000
	3	Trace	0.05 c c pos.	Neg.	Scars on penis and prepuce	0455543210
	4	Neg.	0.01 c c pos.	Neg.	Facial paralysis	1112343200
	5	Trace	0.08 c c pos.	Neg.	Depigmented spots on scrotum and ulcers on penis	0012200420
	6	Neg.	0.07 c c pos.	0.25 c c 1+ 0.5 c c anti-complementary	Numerous skin eruptions and posterior paralysis	0014520021
	7	Neg.	0.03 c c pos.	Neg.	Ulcers on ext. genitalia, incoordination of hind legs	0012344222
Horses giving fixation but no clinical symptoms of Dourine—6	1	Trace	0.02 c c pos.	0.25 c c 4+	None	0000000000
	2	Pos.	0.005 c c pos.	0.1 c c 1+ 0.25 c c 4+	None	0001100000
	3	Trace	0.02 c c pos.	0.1 c c neg. 0.25 c c 4+	None	0001110000
	4	Pos.	0.03 c c pos.	0.1 c c 2+ 0.25 c c anti-complementary	None	0001110000
	5	Neg.	0.01 c c pos.	0.1 c c neg. 0.25 c c 4+	None	0011311100
	6	Trace	0.07 c c pos.	0.1 c c neg. 0.25 c c 2+	None	0001222511
Horses without clinical symptoms, the spinal fluid not giving the fixation test—18	1	Neg.	0.005 c c pos.	Neg.	None	0000110000
	2	Trace	0.02 c c pos.	Neg.	None	0000200000
	3	Neg.	0.005 c c pos.	Neg.	None	0001210000
	4	Neg.	0.1 c c pos.	Neg.	None	1112210000
	5	Trace	0.01 c c pos.	Neg.	None	0012321100
	6	Neg.	0.08 c c pos.	Neg.	None	0155432100
	7	Neg.	0.06 c c pos.	Neg.	None	0355320000
	8	Pos.	0.01 c c pos.	Neg.	None	0000011100
	9	Trace	0.07 c c pos.	Neg.	None	0000011110
	10	Neg.	0.005 c c pos.	Neg.	None	0012333411
	11	Neg.	0.2 c c pos.	Neg.	None	0001012000
	12	Neg.	0.005 c c pos.	Anticomplementary	None	0001203100
	13	Neg.	0.02 c c pos.	Neg.	None	0013332140
	14	Neg.	0.05 c c pos.	Neg.	None	0000120550
	15	Neg.	0.005 c c pos.	Neg.	None	0124111000
	16	Neg.	0.02 c c pos.	Neg.	None	0014413110
	17	Neg.	0.2 c c pos.	Neg.	None	0124411100
	18	Neg.	0.005 c c pos.	Neg.	None	4111222111
Colloidal gold test neg.—2	1	Neg.	0.03 c c pos.	Neg.	None	0000000000
	2	Trace	0.2 c c pos.	Neg.	None	0000000000
Normal control	..	Neg.	Neg.	Neg.	None	0000000000

Of the 7 horses showing clinical evidence of dourine, 1 was markedly positive for globulin, 2 showed a trace and 4 were negative. The 3 showing the presence of globulin were negative to the fixation test. Of the 4 which were negative for globulin, 1 showed a 4+ fixation in 0.25 cc of fluid, a 1+ in 0.25 cc, while the remaining 2 were negative to the test.

As it was impracticable to procure the cords from these horses for microscopic examinations, it is not possible to place conclusive interpretations on the reactions to the colloidal gold test but, in passing, attention is called to the similarity of the reactions in Nos. 1, 2, 3, and 4 to those which might be suggestive of syphilis in man and of No. 5 to a curve as indicative of cerebrospinal syphilis (Kaplan⁶), while that of No. 7 is somewhat similar to the reaction obtained in cases of meningitis in man.

In the 6 tests, of spinal fluid from horses presenting no visible lesions but giving various degrees of fixation, 2 were markedly positive for globulin, 3 slightly so and 1 negative. Of those positive for globulin, 2 gave 4+ fixations with 0.25 cc of fluid, 2 gave 2+ fixations with the same amount while 1 gave a 1+ with 0.1 cc. No. 5, which was negative to the globulin test, gave a 4+ fixation with 0.25 cc of fluid. No. 1, which gave a 4+ fixation and showed a trace of globulin, failed to react in any degree to the colloidal gold test. Were the peaks higher in Nos. 2, 3, 4, and 5, the reactions would be similar to those obtained in cases of spinal syphilis. No. 6 gave a curve not unlike that of meningitis, the reduction peak being almost to the extreme right.

In the case of the horses whose spinal fluids failed to react to the complement-fixation test, the donors presenting no visible lesions of dourine, 2 were positive for globulin while 3 showed a trace; the remaining 13 were negative. In 17 the spinal fluid was negative to the complement-fixation test and in 1 it was anticomplementary. It is interesting to note that the 5 which were positive for globulin, are rather subdued in their reactions to the colloidal gold test. Nos. 2 and 3 have their peaks at 2 on the scale, and in the center; No. 5 has its peak at 3 and in the center; while Nos. 8 and 9 rise to 1, are at the right of the center, and produce nearly the same degree reaction.

Of the 2 fluids that gave no reaction to the gold, one showed a trace of globulin. Whether there were alteration of the cord and

⁶ Serology of Mental and Nervous Diseases, 1914.

meninges is not known. According to Wood, Vogel, and Famulener,⁷ a normal spinal fluid always gives a straight line, but a straight line does not necessarily indicate that the cord is normal, for myelitis in man has given 10 zeros.

SUMMARY

The colloidal gold test, when applied to the spinal fluids of horses suffering with dourine, in many instances gives reactions of varying intensity. A number of the reactions are similar to those in cases of cerebrospinal syphilis in which the peak of the curve is found at about the center of the scale. In some instances, the reactions recorded attained a height of 5. In other cases the reactions showed a tendency to rise and stopped abruptly at 1, 2 and 3, then dropped to zero. This suggests the possibility that a more energetic curve might have been attained had the disease progressed to a greater degree before the destruction of the animal. Some of the curves are similar to those in meningitis, while no reactions of the paretic type were obtained.

While reactions were obtained with the colloidal gold test, just what they may indicate cannot now be stated in the absence of careful study of the spinal cords. However, in the presence of a positive serum test and in some cases of clinical evidence, it would appear that the reactions would be of some significance.

In many cases there appeared to be no agreement between the serum fixation, fluid fixation and the globulin and colloidal gold tests; but when consideration is given the active campaign against this malady and the rapid destruction of animals reacting to the serum fixation tests, and the remote chance of the disease progressing to any great length, it is not surprising that so few reactions to the fixation tests were obtained with spinal fluid. The apparent absence of globulin in many cases is not so disconcerting, as the test employed may not have recorded its presence sufficiently to make it discernible macroscopically; further, other proteins, not detected by the method at hand, may have influenced the colloidal gold reactions.

Several specimens of spinal fluid gave the complement-fixation test for dourine.

⁷ Laboratory Technique, 1917.

ENCAPSULATED NONGAS-FORMING BACILLI

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In 1882, Friedländer found in the sputum of pneumonia patients an encapsulated bacillus to which he gave the name *Bacillus pneumoniae*. Since that time our knowledge concerning organisms characterized by capsule formation has been extended until at present we have three well-defined types of encapsulated bacilli, namely, *B. pneumoniae*, *B. acidilactici*, and *B. lactis-aerogenes*. There is still much uncertainty concerning the characteristics of the subgroups of these organisms. Practically all investigators who have attempted to classify these bacilli have reported irregularities in the cultural reactions. Each worker has presented one or more strains which failed to show cultural reactions corresponding throughout with any member of the fixed groups.

With few exceptions the organisms of this class hitherto described produce acid and gas on one or more of the various carbohydrates. Thus, Strong¹ and Coulter² dealt only with the gas-forming types from numerous sources. Herrold and Culver³ reported 16 strains of gas-forming encapsulated bacilli in a series of 86 cases of renal infections. Meyer and Hinman⁴ reported an encapsulated nongas-forming hemoglobinophilic bacillus from a patient suffering from double hydronephrosis. Perkins⁵ working with cultures from different localities divided his bacilli into 3 groups: (1) those that fermented all the carbohydrates used; (2) those that fermented all but one or two carbohydrates used; (3) those that fermented none of the carbohydrates. In the last group he placed *B. rhinoschleromae*, an organism identified by him as Hopkins culture No. 5, and an organism which he had received from Král's Laboratory. The last culture fermented the carbohydrates after passage through an animal. This observation leads one to question whether the author actually recovered the same organisms which he injected, or one of the other encapsulated organisms which are known to inhabit the intestinal tract of both man and animals.

A review of the literature has revealed no reports of encapsulated nongas-forming bacilli other than those cited. Recently, we have

Received for publication April 1, 1922.

¹ *Centralbl. f. Bakteriöl.*, I, 1899, 25, p. 49.

² *Jour. Exper. Med.*, 1917, 26, p. 763.

³ *Jour. Infect. Dis.*, 1919, 24, p. 114, and *Jour. Am. Med. Assn.*, 1918, 70, p. 1444.

⁴ *Ibid.*, 1920, 27, p. 72.

⁵ *Ibid.*, 1904, 1, p. 241.

isolated from different sources three organisms of this description. This paper is a report of the morphologic, cultural, and immunologic characteristics of these organisms.

Culture No. 1 was isolated from a white man, aged 44, who had always enjoyed good health until a short time before he came to the genito-urinary clinic for treatment. He gave a history of having had the usual symptoms of an acute cystitis. These acute symptoms had subsided before the patient applied for treatment. He was somewhat annoyed by the continuation of the cloudiness of the urine and by the offensive odor given off by the urine at the time of micturition.

On cystoscopic examination the patient presented a chronic cystitis. A specimen of urine removed from the bladder by catheter was extremely turbid and had a foul odor similar to that produced by decomposing protein material. Examination showed a heavy trace of albumin, many pus cells, a few red blood cells, and many nonmotile bacteria. Culture of this specimen revealed a small gram-negative, encapsulated, nongas-forming, aerobic bacillus. Cultures of urine from this patient at three different times during the next four months showed the same organism.

Culture No. 2 was isolated from a white woman, aged 30 years, who entered the hospital complaining of periodic attacks of abdominal pain. The trouble dated back to her first pregnancy about 8 years ago. The patient said that she had had attacks of severe abdominal pain at intervals of approximately 6 months, since the onset of the trouble; that there was pain on urination; and that during these attacks the urine was always bloody. There was a contracted urethral meatus which required mechanical dilatation before a cystoscopic examination could be satisfactorily accomplished. The findings were a pyelitis of the left kidney and a normal bladder. Specimens of urine were obtained from the bladder and left ureter. They showed a moderate amount of albumin, many pus cells, and a few red blood cells. Cultures of the urine from the left ureter and the bladder contained a small gram-negative, encapsulated, nongas-forming, aerobic bacillus.

Culture No. 3 was obtained at necropsy from the left pleural cavity of a white woman, aged 30 years, who had been complaining for 12 years or more of "kidney trouble." One year before death the urine was found heavily loaded with albumin, and she was refused an operation which previously had been advised. Several months later, she was admitted to the medical service of the Allegheny General Hospital, where the diagnosis of lobar pneumonia, endocarditis, and uremia was made. Just prior to admission she developed subcutaneous ecchymoses and considerable bleeding from the nose and gums. She died 8 days after admission. Necropsy revealed: acute and chronic interstitial and parenchymatous nephritis; chronic endocarditis, myocarditis, and pericarditis; arteriosclerosis; bronchopneumonia, acute and chronic pleurisy with effusion; subconjunctival and palpebral ecchymosis; healed tubercles of the lung, liver and spleen; and anasarca. The left pleural cavity contained a yellow flaky fluid from which was cultivated streptococcus hemolyticus and a small gram-negative, encapsulated, nongas-forming, aerobic bacillus similar to those already described.

DESCRIPTION OF THE ORGANISM

The organism under discussion is a gram-negative, nonspore-bearing, encapsulated, nonmotile rod which varies in length from 1 to 2

mikrons—and in diameter from .3 to .5 mikrons, in general being a little smaller than the typhoid bacillus. The capsules differ somewhat in their size and staining characteristics. In the first culture they are demonstrated easily from any type of medium, while in the other two cultures they are demonstrated only when grown in milk. The relative size of the capsules compares with the thick capsules of the *B. lactis-aerogenes* type somewhat as the capsules of pneumococci types 1, 2 and 4 compare with the capsules of type 3; that is, the capsules of these organisms stain more faintly, appear to be smaller, and the bacilli occupy a relatively smaller amount of the capsule than those of *B. lactis-aerogenes*. On gelatin and agar the colonies vary from thin translucent coli-like colonies of medium size to large thick slimy mucoid *aerogenes*-like colonies. They grow best at 37 C. and more slowly at 20 C. The cultural reactions of the organisms obtained from the 3 sources described correspond throughout with few exceptions. In table 1 the cultural characteristics of each, observed over a period of 3 weeks, are recorded. It shows their similarity and the slight points of variation which exist between them.

If the results recorded are examined in relation to the types of carbohydrates used, several interesting points will be noted.

1. Monosaccharids:

(a) Pentoses	{ arabinose rhamnose }	acid, no gas
	xylose	no acid, no gas
(b) Hexoses	{ dextrose mannose galactose levulose }	acid, no gas
	inosite	no acid, no gas

2. Disaccharids:

maltose	acid, no gas
{ lactose sucrose }	no acid, no gas

3. Trisaccharids:

raffinose	no acid, no gas
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4. Polysaccharids:

dextrin	acid, no gas
{ inulin salicin }	no acid, no gas

5. Alcohols:

{ mannitol dulcitol sorbitol }	acid, no gas
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6. Glycerol:

glycerol	no acid, no gas
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TABLE 1
CULTURAL CHARACTERISTICS *

Cultures	Dextrose	Levulose	Galactose	Maltose	Mannitol	Xylose	Arabinose	Rhamnose	Sorbitol	Dulcitol	Lactose	Saline	Sucrose	Raffinose	Inositol	Dextrin	Mannose	Inulin	Glycerol	Milk	Gelatin	Indol	Lead Acetate	Motility	Pathogenic
Culture 1:																									
First day.....	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Third day.....	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Fifth day.....	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Tenth day.....	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Fourteenth day..	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Twenty-first day	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Culture 2:																									
First day.....	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Third day.....	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Fifth day.....	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Seventh day.....	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Tenth day.....	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Fourteenth day..	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Twenty-first day	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Culture 3:																									
First day.....	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Third day.....	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Fifth day.....	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Tenth day.....	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Fourteenth day..	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+
Twenty-first day	ac	ac	ac	ac	ac	ac	ac	ac	ac	ac	t	t	t	t	t	ac	ac	ac	ac	ac	ac	+	+	+	+

ac = acid; alk = alkaline; sl = slightly; str = strongly; pep = peptonization; dec = decolorized; t = turbid; ac = no change; + = positive; - = negative.
 * In order to be certain that the carbohydrates were reacting properly, we cultivated a series of control organisms parallel with the unknown strains. Those used as controls were: B. lactis-aerogenes, B. coli-communior, B. typhosus, B. paratyphosus A and B, and B. dysenteriae; and each strain gave its typical reaction on each of the carbohydrates.

It is to be noted that the organisms show no particular preference for any group of carbohydrates. They produce acid on 2 pentoses, 4 hexoses, 1 disaccharid, 1 polysaccharid and 3 alcohols. They fail to produce acid on 1 pentose, 1 hexose, 2 disaccharids, 1 trisaccharid, 2 polysaccharids, and 1 glycerol.

The production of indol is constant with the three cultures.

The reactions on litmus milk are the most striking and characteristic cultural findings. In the first 3 days there is no change. On the third day the medium is decolorized or develops a chalky appearance which lasts until the sixth day. At this time a slight trace of alkalinity develops; and on the seventh day a definite alkalinity is noted which gradually increases to a maximum reached on the tenth day. Coagulation does not occur. The casein of the milk is peptonized after the appearance of the alkaline reaction, the medium gradually loses its opacity and becomes a transparent deep blue. It is noted that when the tubes of litmus milk are inoculated and immediately sealed, or sealed within 24 hours, the change in the litmus milk proceeds only to the stage of decolorization, and that neither the peptonization nor secondary alkalinity develops. This suggests that perhaps all of the available oxygen in the tube has been consumed and that there is not a sufficient amount remaining to permit the complete reaction described to take place as it does when the tubes are not sealed.

It will be noted on examination of table 1 that the cultural reactions of these bacilli are essentially the same as those of *B. dysenteriae* (Flexner). The Flexner organism, however, does not possess a capsule and is less alkaline on litmus milk.

PATHOGENICITY

Each of the 3 strains when first isolated was highly pathogenic for guinea-pigs and rabbits, but has become less pathogenic after prolonged artificial cultivation. When first isolated 1 cc of a 24-hour broth culture given intravenously, killed, in 30 minutes, a rabbit weighing 2 kilograms; but when given intraperitoneally, it required 24 hours to kill a rabbit of similar weight. Necropsy on these animals showed an acute diffuse peritonitis similar to that produced by *B. coli*. Similar reactions were obtained with guinea-pigs. It was also found that 1 cc of the 24 to 48 hour broth culture when freed from the bacilli, contained sufficient toxic material to produce a severe reaction in rabbits almost instantly.

IMMUNOLOGIC REACTIONS

The serum from the first patient agglutinated the organism isolated from him, in dilutions varying from 1/10 to 1/160, while the serum from the second patient did not agglutinate either the organism obtained from her or that obtained from the first patient. No tests were made with the serum of the third patient as the culture was obtained at necropsy.

Rabbits were immunized against each of the 3 cultures. Immunization was started by injecting intravenously small doses of the organisms suspended in saline and heated for 30 minutes at 56 C. For subsequent injections the time of heating was gradually decreased until it was possible to give living organisms intravenously without apparent damage to the animals.

TABLE 2
AGGLUTININ TITERS

Bacilli	Antiserums				
	Anti-serum 1	Anti-serum 2	Anti-serum 3	Anti-dysentery (Flexner) Serum	Antiserums for Typhoid, Paratyphoid, Dysentery (Shiga, Hiss-Y, Rosen), Enteritidis Bacilli
Bacillus 1.....	400	0	1600	50	0
Bacillus 2.....	50	50	50	50	0
Bacillus 3.....	100	100	100	50	0
Typhoid, paratyphoid, dysentery (Flexner, Shiga), pseudodysentery, lactis aerogenes bacilli.....	0	0	0		

The figures give the highest active dilution of the antiserum in each case.

Ten days after the last injection the animals were bled and the serum of each was tested against the three strains in question, against *B. typhosus*, *B. paratyphosus* A and B, *B. dysenteriae* (Flexner), *B. dysenteriae* (Shiga), a pseudodysentery bacillus, and *B. lactis-aerogenes*.

Table 2 shows that the animals immunized against 2 of the organisms developed strong agglutinating power for their homologous strains and somewhat less agglutinating power for the other 2.

In order to compare fully these organisms serologically with the typhoid-dysentery group, emulsions of the three types of bacilli were tested with serum against the following organisms: *B. typhosus*, *B. paratyphosus* A and B, *B. dysenteriae* (Shiga), *B. dysenteriae* (Flexner), *B. dysenteriae* (Hiss-Y), *B. dysenteriae* (Rosen), and *B. enteritidis* (Gartner).

It is interesting to note (table 2) that the only serum which agglutinates these bacilli at all is that against *B. dysenteriae* (Flexner). These reactions added to those on the carbohydrates indicate that there is a close relationship between the encapsulated bacilli described in this report and the *B. dysenteriae* (Flexner).

DISCUSSION

In reviewing the literature on encapsulated gram-negative bacilli, we realize the need of a more thorough and systematic study of this group which Winslow, Kligler, and Rothberg⁶ have designated as "an extraordinarily variable group." These authors have given an admirable classification of the colon-typhoid group of bacteria, but seem to have been content to pass by the encapsulated group with a short description of *B. aerogenes* (Escherich) and a few words about the irregular forms of this group.

Other authors,⁷ make more or less limited reports, not taking up in detail the common irregularities which one so frequently encounters in the routine work of a large hospital. We believe that bacteriologists are beginning to realize the need of more detailed knowledge of this group of organisms, particularly since they are so frequently found in infections of the genitro-urinary tract. On account of their close resemblance to the colon bacilli in morphology, staining and cultural reactions, it is possible that they are not infrequently mistaken for colon bacilli.

If such work as that of Winslow, Kligler, and Rothberg⁶ can be extended to other groups of organisms, we shall be able to avoid much of the confusion that has arisen and continues to arise among the bacteria which are so variable.

It is interesting to note the reports of encapsulated forms of *B. paratyphosus* B, and *B. dysenteriae* by Fletcher⁸ which the author states were made without a complete investigation of the organisms, because the military service made it impossible for him to give sufficient time to complete the work properly. He also states that from peptone water he could obtain both encapsulated and nonencapsulated forms. We would suggest, therefore, that probably the author was not dealing with a pure culture of *B. paratyphosus* B, or *B. dysenteriae*, but that he probably had a mixed culture of *B. paratyphosus* B and

⁶ Jour. Bacteriol., 1919, 4, p. 429.

⁷ Levine: Jour. Am. Public Health Assn., 1917, 7, p. 784. Fitzgerald: Jour. Infect. Dis., 1914, 15, p. 268

⁸ Lancet, 1918, 2, p. 102.

a slow lactose fermenting *B. acidi-lactici* which is also encapsulated. We have isolated one culture of this type which did not ferment lactose until the fourteenth day.

The organisms we describe are apparently closely related to the organism described by Meyer and Hinman, and yet there are several points of difference. Their organism was hemoglobinophilic, grew in small mucoid colonies, and when smears were made from the growth with a platinum loop, the whole colony was frequently removed. Our bacilli, on the other hand, are not hemoglobinophilic, they grow readily on all ordinary culture mediums and the colonies resemble under some conditions, typical *B. coli* colonies, and under others, typical *B. lactis-aerogenes* colonies. It is practically impossible to remove the whole colony at one time with the platinum loop. The cultural reactions, however, are almost identical with those obtained by Meyer and Hinman.

If the bacilli which we describe here belong in the colon-typhoid group of bacilli, they should be placed in group 2, according to the classification of Winslow, Kligler, and Rothberg, along with *B. dysenteriae* (Flexner). There is a close relationship culturally and an apparent one immunologically between the two. If they do not belong in the colon-typhoid group of bacilli, we believe that *B. pneumoniae*, *B. acidi-lactici*, and *B. lactis-aerogenes* should also be removed from that group and that all of the encapsulated bacilli should be placed in a group separate and distinct from the colon-typhoid group. The organism we describe seems to occupy a position among the encapsulated bacilli similar to that occupied by the *B. dysenteriae* (Flexner) in the typhoid-paratyphoid-dysentery group.

SUMMARY

A previously unrecognized organism, a small gram-negative, encapsulated, nongas-forming, aerobic bacillus was found in three patients, each of whom was suffering from some pathologic condition of the genito-urinary tract. It was not possible to determine whether the organism was the primary etiologic factor in producing the lesions or a secondary invader.

THE SPECIFIC PRECIPITIN REACTION OF THE NORMAL AND CATARACTOUS LENS

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In this article are recorded briefly the results of experiments on the precipitin reaction of the lens,¹ with special reference to the human lens in senile cataract.

It is a remarkable fact, discovered by Uhlenhuth and verified by others,² that the lens of different species contains identical antigenic elements. A lens antiserum produced by injecting a rabbit or guinea-pig with beef lens reacts in precipitation, anaphylaxis and fixation tests not only with beef lens but also with the lens of other mammals, of birds and amphibians. With fish lens the precipitin reaction, however, is faint. The specificness of the reaction is limited not by species as in the immune reactions of blood, of serum proteins, and of bacteria, but by the organ from which the antigen is derived.

I have studied the precipitins that develop in rabbits on the injection of lens solutions in 0.9% salt solution. The principal experiments have been made with approximately 5 or 10% solutions by weight of beef, horse, rabbit, sheep and swine lens, removed with special care to avoid admixture with blood or serum, and with approximately 2.5% solutions of human lens, the seat of senile cataract. The preliminary tests of cataractous material were made with 5 lenses from the Cook County Hospital (Dr. E. V. L. Brown). The 50 cataractous lenses in table 1 were obtained from the Illinois Charitable Eye and Ear Infirmary (Dr. E. K. Findlay). In all cases the solutions of lens substances have been made by shaking lenses with glass beads in 0.9% salt solution. The strength of the resulting solutions is based on the weight of the original lens, and as the amount of insoluble residue varies greatly, especially in cataractous lenses, the strengths of the lens solutions given in this article are as a rule approximate only. For injections and tests absolutely clear lens solutions have been used. In some cases the lens solutions were passed through Berkefeld filters in order to insure sterility. Four

Received for publication April 1, 1922.

¹ A brief report of the observations on the precipitin reactions of the normal lens appeared in Jour. Am. Med. Assn., 1921, 77, p. 32, and Trans. Chicago Path. Soc., 1921, 11, p. 221.

² The literature is reviewed by Kodama, Jour. Infect. Dis., 1922, 30, p. 418.

differences between the antigens in beef lens and human lens of senile cataract or that the solutions of cataractous lenses may contain substances that inhibit the precipitin reaction of antiserum for beef lens. This statement seems applicable also to solutions of the fetal human lens. Several attempts were made by specific absorption methods to obtain indications of different precipitinogens in cataractous and beef lens solutions and of different precipitins in the respective antisera, but invariably treatment of any lens antiserum with solutions of cataractous or other lens and of anticataract serum with solutions of beef or other lens resulted in the complete removal of all the antilens precipitins. So far then, no differences have been observed in the precipitin reactions that seem to point to a possible separation into distinct groups of the cataractous lenses, all of which were classed as examples of senile cataract by the ophthalmologists. Whether other and rarer forms of cataract are associated with more definite alterations in the precipitinogenic nature of the lens protein is an interesting question. To answer such questions with success it no doubt will be of great advantage to use antilens sera of as nearly monovalent or simple a nature as possible rather than the presumably more complex antisera from injections of solutions of many different lenses probably containing a greater variety of more or less closely related antigens.

The absorption experiments referred to were made by mixing the antiserum in full strength with one or two volumes of lens solution, varying in strength from 1:100 to 1:600, leaving the mixtures at room temperature for an hour or two and then in the icebox over night. The precipitate that had formed was removed by centrifugation and the clear fluid tested. Similar experiments with lens antiserum and dilutions of normal serum have given negative results, that is, the lens precipitin was not removed.

The lenses of puppies 3 weeks old reacted like the lenses of adult dogs. Fetal human lenses from all stages (obtained from the Cook County Hospital through Dr. Willson B. Moody), the earliest being at about the end of the second month, all gave typical lens specific reactions, but in much higher dilutions with antisera for human lens (cataractous) than with antisera for beef lens, for instance, as shown in table 2, this result suggesting as stated already the presence in the fetal lens either of antigens that differ from those in the beef lens or of substances that inhibit the action of beef lens precipitin. Other possibilities may be suggested also. In two of the fetal human lenses ordinary antihuman serum showed the presence of species-specific

proteins but only in small quantities, a result that appears to differ somewhat from that of Szily³ who found species-specific proteins in considerable quantities regularly in the embryonal chicken lens. No doubt a further study of the embryonal lens by means of the precipitin reaction would yield interesting results.

Extracts of beef retina, uvea, and cornea gave no reactions with strong antiserum for beef lens and the serums of rabbits injected with extracts of beef cornea and of beef uvea gave no precipitin reactions with beef lens or any of the other lenses, both these serums reacting strongly, however, with beef serum and extracts of beef cornea. The antiuveal serum reacted well with extract of beef uvea and retina also, but the anticornea serum was not tested with these extracts. These results invite further study with the precipitin reaction. As pointed out previously,¹ the normal aqueous and vitreous humors, which contain species-specific antigens, may give a precipitin reaction with lens antiserum under conditions that seem to preclude the passage of this substance into the humors as the result of handling of the eye. The aqueous humor of the eye of a rabbit, from which the lens had been removed by the capsular method one month before (Dr. R. Kodama), gave a strong reaction with antisera for beef and human lens. At present the significance of the occasional presence in the ocular humors of substances that react with antilens serum must remain unexplained. One fact stands out—the lens is much more strictly specific in its precipitin reactions, with reference to other eye tissues, than in its anaphylactic reactions, as shown in the recent work of Kodama².

The injection of strong antilens serum into the aqueous or vitreous in rabbits, after first withdrawing the proper quantity of humor, did not produce any peculiar or specific action on the lens.

In all cases the serum of rabbits containing lens precipitins, as the result of the injection of heterologous lens solutions, caused precipitates in solutions of rabbit lens in seemingly just as high dilution as in any other lens solution. Several rabbits have been injected many times intravenously and intramuscularly with solutions of rabbit lens, but lens precipitins could not be demonstrated in the serum. In other rabbits the injections of rabbit lens were associated with injections of sheep serum in order to put the antibody-forming mechanisms in motion, but no lens precipitins developed. In rabbits previously injected with beef or human lens, the injection of rabbit lens at a time when the

³ Klin. Monatsbl. f. Augenheilk., 1921, 12, p. 150.

precipitins from the first injections had disappeared from the blood almost completely, in one or two instances seemed to cause the production of small quantities of lens precipitins which, however, were lost quickly. In one rabbit previously injected with solution of human cataractous lens, in consequence of which it became for a time an active producer of lens precipitins, reinjection with solution of rabbit lens, which had been passed through a Berkefeld filter, was followed by a definite and well sustained new production of lens precipitins acting on solutions of rabbit as well as of other lenses. Contrary to the results of certain other investigators, Römer and Gebb ⁴ as well as Morax and Pollack ⁵ found that the guinea-pig reacted typically to the injection of guinea-pig lens only after sensitization with the lens of a different species. Similarly, rabbits, as a rule, appear to produce precipitins that react with rabbit lens as the result only of immunization with the lens of a different species. The problems presented by this form of "Horror autotoxius" challenge renewed attack. In connection with this matter, it is of great interest to note that Guyer ⁶ after many attempts succeeded "in securing a defective-eyed young rabbit from a mother of normal stock by injecting her repeatedly with pulped rabbit lens before and during pregnancy."

SUMMARY

Precipitin tests of the lens of different species—beef, chicken, dog, guinea-pig, human (normal and cataractous), monkey, rabbit, rat, sheep, swine—with antisera for beef, horse, human cataractous, sheep and swine lens, reveal a consistent organ-specificity in the precipitin reaction. The normal lens of these mammals gives no indications in the usual tests of containing any species-specific precipitinogens. This is true also of the lens of senile human cataract with the exception that 8 of 50 cataractous lenses gave a reaction for human serum albumin. In some cases the fetal human lens may contain species-specific elements.

The lens-specific elements in normal, cataractous and fetal lenses probably are not precipitinogenically identical, and it is possible that solutions of cataractous and other lenses contain substances that interfere with the action of heterologous antilens precipitins. Studies with monovalent or simple antilens serum may help to solve questions of this kind.

⁴ Arch. f. Ophth., 1912, 81, p. 367.

⁵ Ann. de l'Inst. Pasteur, 1914, 28, p. 625.

⁶ American Naturalist, 1921, 55, p. 97.

The aqueous and vitreous humors sometimes may contain substances that react with antilens serum. Beef cornea, retina and uvea, however, do not appear to contain any precipitinogens in common with the lens.

Under ordinary circumstances rabbits produce precipitins that react freely with solutions of rabbit lens in response only to injections of lens of different species and not in response to injections of rabbit lens. Rabbits previously injected with foreign lens material, that is rabbits rendered allergic with respect to the lens, however, may respond to injection of solutions of rabbit lens with new production of lens precipitins which act on rabbit as well as other lens solutions.

DEVELOPMENT OF PARATYPHOID-ENTERITIDIS GROUP IN VARIOUS FOODSTUFFS

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In the study of so-called "food poisoning," more properly termed food infection, by members of the paratyphoid-enteritidis group, it was deemed advisable to gain some idea of the ability of the various members of this group to multiply in miscellaneous food products. Numerous instances are on record in which milk and certain meat products, such as meat pies, the English brawn, meat soups and gelatin have served as excellent culture mediums for these forms, with resultant "food poisoning" outbreaks. The summary given by Savage¹ of the outbreaks of "food poisoning" in England shows that while meat products comprise the class of food most commonly incriminated, others have occasionally been held responsible. In the present work various vegetable foods and fruits as well as several meat products were used. In each case the aim has been to determine, at several different temperatures, the rapidity of development and the ability to spread from the point of inoculation throughout the foodstuff.

Another phase of this subject which seems important from the standpoint of prevention is that of the physical condition of the infected food. May foods in which members of the Gaertner group have had an opportunity to multiply exhibit physical evidence of spoilage to such an extent that it would be forced on the consumer's attention and cause him to reject the food as unfit for consumption? Since many of the past reports of outbreaks following food infection have failed to record information bearing on this point, it has been given some attention in the present investigation.

The question of the possible production by these organisms of soluble toxin in various foodstuffs is of paramount importance, but it could not be included in the present investigation, as no strains were available which consistently caused illness when fed to experimental animals.

As a rule, canned foods, particularly vegetables and fruits, were used as the source of test materials, since the fresh products were not

Received for publication April 7, 1922.

¹ Food Poisoning and Food Infections, 1920, p. 76.

available at all times. The hydrogen-ion concentration of such canned products is greater than that of the corresponding unheated foods,² and it is possible that in some cases at least the use of fresh materials might have given different results. The procedure here followed, however, would simulate closely conditions in the household where after the partial consumption of cooked food the remainder is set aside for future use. The foods to be inoculated were contained either in flasks or in large glass dishes, according to the nature of the product. When working with liquid or semiliquid foods a measured quantity, usually 50 or 100 c.c., was introduced into 300 c.c. Erlenmeyer flasks and sterilized in the autoclave for 30 minutes at 15 pounds pressure. In some instances certain modifications of this procedure were necessary, for example, in the case of evaporated milk and the various fruits which are packed in sugar syrups. These were removed aseptically from the original can in order to avoid sterilization. Solid substances, such as meat, were held in large glass dishes. Aerobic conditions were maintained throughout the work.

Inoculation was made from a 24-hour broth culture diluted to such an extent that on the introduction of one loopful the test substance should contain only a few thousand organisms per c.c. When testing the ability of the paratyphoid group to develop and spread in solid materials, such as cooked meat, inoculation was made by placing one loopful of a diluted broth culture at one spot on the surface and then, with a straight wire, stabbing through this well into the material. By this procedure the greater number of organisms in the inoculum were left on the surface, while a few were carried into the interior of the mass. In every case tubes containing a known amount of sterile water were also inoculated and an estimate of the numbers of cells in the inoculum was obtained by plating.

Specimens were held at several different temperatures, 37 C., 20 C., and in an icebox the temperature of which varied from 6 to 9 C. At regular intervals observations were made on the physical condition of the food, and samples were withdrawn for plating and for determination of the hydrogen-ion concentration. The hydrogen-ion concentration was determined only roughly by mixing in the depressions of a porcelain test plate 0.5 c.c. of the food product with one drop of a suitable indicator. Dextrose litmus agar was used for plating. Wherever possible the plate counts are expressed as organisms per c.c. of the liquid part of the food product. The solid foods were examined by removing

² Bigelow, W. D., and Catheart, P. H.: National Canners Assn. Bull. 17-L, 1921.

small pieces of the material—about 1 gm.—weighing and grinding with sterile sand in a mortar. This was followed by dilution and plating. The location and distance from the site of inoculation of the removed pieces was always recorded.

On examination of the cooked meats, which had not been sterilized previous to inoculation, it was found that the paratyphoid colonies were sufficiently distinct in appearance on dextrose litmus-agar plates so that

TABLE 1
DEVELOPMENT OF *B. ENTERITIDIS* (BRIGHTON) IN SOME MISCELLANEOUS FOODSTUFFS

Temperature of Incubation	No. of Samples	Original Inoculum	Plate Counts After				PH	
			24 Hours	48 Hours	4 Days	7 Days	Before Inoculation	At 7th Day
Sauerkraut:								
23° C.	1	1200*	Sterile	Sterile	Sterile	3.4	
20° C.	1	1200	Sterile	Sterile	Sterile		to	
6-9° C.	1	1200	Sterile	Sterile		3.6	
Tomatoes:								
37° C.	2	2650	27,000,000*	49,000,000	26,000	Sterile	4.0	4.0
20° C.	2	2650	90,000	800,000	18,000,000	19,800,000	to	to
6-9° C.	2	2650	3,800	7,000	4,500	1,500	4.4	4.4
Spinach:†								
37° C.	2	2400	144,000,000	3,900,000	Sterile	Sterile	About	5.2
20° C.	2	2400	19,000	3,200,000	52,000,000	1,300,000	5.2	to
6-9° C.	2	2400	2,600	2,100	2,200	4,200	to	5.4
String beans:†								
37° C.	2	1900	40,000,000	14,000,000	10,000	Sterile	5.2	5.0
20° C.	2	1900	520,000	195,000,000	165,000,000	155,000,000	to	5.0
6-9° C.	2	1900	1,400	1,500	630	5.4	5.3
Corn:								
37° C.	2	2300	216,000,000	2,460,000	Sterile	Sterile	5.8	5.0
20° C.	1	2300	66,000,000	380,000,000	230,000,000	158,000,000	to	5.0
6-9° C.	2	2300	9,600	11,000	95,000	6.2	6.0
Peas:								
37° C.	2	2800	3,200,000,000	5,900,000,000	2,900,000,000	350,000,000	5.8	8.6
20° C.	2	2800	4,000,000	1,200,000,000	2,500,000,000	4,000,000,000	to	7.6
6-9° C.	2	2800	7,000	37,000	110,000	6.0	6.0
Evaporated milk:								
37° C.	2	7000	1,500,000,000	3,400,000,000	1,200,000,000	630,000,000	5.2	7.2
20° C.	2	7000	340,000	230,000,000	4,200,000,000	3,700,000,000	to	6.6
6-9° C.	2	7000	5,600	6,800	25,000	5.4	5.4

* Counts are expressed as numbers per c.c. of the liquid portion of the food product.

† Several additional samples of spinach and string beans have given discordant results, showing a gradual decline in numbers instead of a multiplication. These samples had a slightly greater hydrogen-ion concentration than those shown in the above table.

no confusion resulted in differentiating them from the miscellaneous organisms surviving the cooking of the meat. As an additional precaution colonies were fished from time to time, transferred to Russell's medium, and agglutination tests were made to check the identity of the cultures thus obtained.

The types of the Gaertner group regularly employed throughout this work were a strain of *B. enteritidis* isolated by Savage and Forbes³

³ Jour. Hyg., 1918, 17, p. 460.

from a human food poisoning outbreak at Brighton, England, and strain "rabbit 1371," a representative of the animal paratyphosus B group, obtained by Litch and Meyer ⁴ during the course of an outbreak among rabbits. Other organisms employed from time to time included the Jordan 210 ⁵ and Rowland strains of human *B. paratyphosus* B, or *B. schottmülleri*, and the Calf-typhus 1 strain described by Ten Broeck. ⁶ The behavior of these various cultures was, as a rule, quite similar, so that the results with one only are presented in detail.

Table 1 shows the development of *B. enteritidis* in various vegetable foods and in evaporated milk. With the exception of the highly acid sauerkraut, this organism is able to multiply, at least to a certain extent, in all of the products tested. The growth in tomatoes was rather surprising in view of the acidity of the product. One of the noteworthy features of the table is the fact that at 37 C. there occurred in many of the products a rapid growth during the first 24 or 48 hours, followed by an abrupt decline in numbers. This was true of tomatoes, spinach, corn, and string beans. In some instances no viable organisms could be found in 1 c c quantities at the fourth day after inoculation. This rapid decrease in numbers following an initial multiplication is correlated with a high hydrogen-ion concentration and occurred in those foods in which the acidity is increased by the metabolic activities of the organism and also in those which maintain during growth an originally high hydrogen-ion concentration. In peas and evaporated milk the change in the hydrogen-ion concentration was toward an alkaline reaction, in contrast to the foodstuffs mentioned. Also, in these 2 products the decline in the number of organisms, after the height of the growth curve was reached, is gradual and not abrupt. Despite the increased percentage of solids in the evaporated milk, the growth of the Gaertner group and the resultant changes in the hydrogen-ion concentration were quite similar to those taking place in the ordinary skim milk medium. Other types of the Gaertner group gave similar results, although the decrease in numbers in the acid foodstuffs was in most cases less abrupt than that exhibited by the Brighton strain of *B. enteritidis*. For example, viable organisms of the "rabbit 1371" strain were frequently present in small numbers after 7 days at 37 C. At 20 C. the rate of growth of all the cultures tested was slower, and the period of maximum development was not followed by the speedy destruction which took

⁴ Jour. Infect. Dis., 1921, 28, p. 27.

⁵ Ibid., 1917, 20, p. 457.

⁶ Jour. Exper. Med., 1920, 32, p. 19.

place at the higher temperature. In the icebox there was a slow multiplication in several of the foodstuffs.

In a number of instances other organisms, such as the colon bacillus, were used together with the paratyphoid strains to inoculate the foods. In such cases *B. coli* grew more rapidly than did the paratyphoids, although the presence of the latter could usually be detected by streaking Endo medium with some of the material in question. The growth of the colon bacillus was accompanied frequently by the production of gas and sour odors in many of the foods, whereas the paratyphoids alone usually gave much less evidence of their presence.

In addition to the food products already enumerated, several fruits were used. Samples of pineapples, raspberries, cherries and pears were inoculated with approximately the same number of organisms as were the vegetables. In every case there was a rapid decline in numbers, and 1 c c quantities of the product frequently yielded negative results after 24 hours. This reduction was most rapid at 37 C. and least so at 6 to 9 C. At the latter temperature small numbers of viable cells would sometimes be detected after 48 hours or more. The several strains differed slightly in their resistance to the destructive effect of the organic acids of the fruits, although this difference was not marked. The hydrogen-ion concentrations of the fruits were: pineapples, P_H 3.4-3.6; pears, P_H 4; cherries P_H 3.8-4; that of the raspberries could not be determined with indicators due to the color of the product. The figures given by Bigelow and Cathcart,² as determined electrometrically, vary from P_H 3.23-3.69.

When determining the ability of *B. enteritidis* to develop in meat products, corned beef, cooked ground meat (Hamburger steak), and canned salmon were used. The detailed results secured with samples held at several temperatures are presented in table 2. Here it is seen that in all of these products, under favorable temperature conditions, there is a multiplication at the point of inoculation and more or less spreading over the surface and throughout the interior. The moisture content and texture of a food quite naturally determine the extent to which the organisms may spread. Thus in a compact, comparatively dry product, such as corned beef, the area throughout which *B. enteritidis* was found during the first period of development was considerably smaller than in the softer foodstuffs containing liquid. A comparison of the results secured at the different temperatures is of interest in connection with the potential danger of contamination of a large mass of food from one original point. Thus, in Hamburger steak, within a period of 12

hours at 37 C., there is a rapid multiplication at the point of inoculation while penetration to other parts of the meat has not occurred. In contrast to this, after 24 hours great numbers of organisms are to be found at some distance from the original point of inoculation. At 20 C., the period of maximum growth at the point of inoculation occurs after the first 12 hours, while even after 48 hours the organisms have failed to spread to any extent throughout the meat. It is evident that, while multiplication may take place at the point of inoculation under unfavor-

TABLE 2
DEVELOPMENT OF B. ENTERITIDIS (BRIGHTON) IN SEVERAL TYPES OF MEAT PRODUCTS

Sample Held at	Original Inoculum	Interval After Inoculation	Location and Approximate Distance from the Point of Inoculation					
			Point of Inoculation *	Surface			Interior	
				1 Cm.	3 Cm.	5 Cm.	1 Cm.	3 Cm.
Corned Beef: 37° C.	4,800	Hrs.						
		24	3,600,000	270,000	0	0	6,000	0
		48	56,000,000	Contaminated	2,700,000	81,000	10,000,000	0
20° C.	4,800	24	5,800	80	0	40	0
		48	18,000	0	0	0
Ham-burg Steak: 37° C.	10,000	12	570,000,000	37,000,000	0	0	21,000,000	210
		24	26,400,000,000	11,400,000,000	230,000,000	180,000,000	17,400,000,000	300,000,000
		48	3,800,000,000	3,000,000,000	1,700,000,000
20° C.	10,000	12	12,200	330	0	20	0
		24	200,000,000	22,400	0	510	11,000,000	0
		48	4,600,000,000	7,400,000	0	24,000,000	21,000
Red Salmon: 37° C.	6,600	12	780,000,000	9,100,000	0	0	60,000,000	3,700
		24	3,500,000,000	3,100,000,000	860,000,000	48,000,000	355,000,000	Contaminated
20° C.	6,600	24	3,600,000	0	0	0	9,300	0
		48	7,700,000	6,000	2,500	0	230,000	40,000
9° C.	6,600	24	1,000	0	0	0	200	0
		48	2,500	0	0	0	600	0
		96	5,900	20	0	0	10	0

* Counts are expressed as number of B. enteritidis per gram.

able temperature conditions, it is only at the optimum temperature that the rapid penetration and spreading of the organisms throughout the meat occurs. Obviously, then, accidentally contaminated meat held at body temperature would constitute a much greater source of danger than if held at a lower temperature, due not merely to the increase in numbers at the point of contamination but especially to the spreading of the organisms throughout the product.

Throughout this work it was observed regularly that in most of the foods the paratyphoid-enteritidis group failed to give any visible evidence of growth. Peas were the most noteworthy exception, for the

organisms when growing in this product formed a thin white surface pellicle. In several of the food products, especially peas and corn, there was some evidence of gas formation. This might readily escape the notice of the consumer, however, unless the gas were prevented from escaping. With the exception of these few cases the contaminated foods regularly appeared normal. Also, no abnormal odors could be detected in any instance. From these observations it may be stated that extensive multiplication of the Gaertner group may take place in most foodstuffs with little or no resultant readily detectable evidence of growth.

A review of the reports of outbreaks of food infection occurring abroad and in the United States has revealed relatively few which make any mention of the physical condition of the food and whether the consumer had any warning that the contaminated food responsible for the outbreak was unfit for consumption. However, the reports which have included this point practically all agree that the food appeared normal at the time of consumption and that there was nothing to excite suspicion as to its quality. Gaertner,⁷ in his original description of *B. enteritidis* and the classic "meat poisoning" outbreak at Frankenhausen, Germany, stated that to all appearances the meat could not be differentiated from normal meat. Kaensche⁸ recorded an extensive outbreak at Breslau attributed to chopped beef contaminated with *B. enteritidis*, and stated that the meat presented no abnormal appearance either in color, odor, or consistency. A few years later Bowes and Ashton,⁹ in the report of an outbreak caused by veal pies contaminated with *B. enteritidis*, mention that "practically no one complained of the taste or appearance of the pies, which had no smell whatever." Corned beef (Barker¹⁰) and soup made from meat (Curschmann¹¹) contaminated with *B. enteritidis* were held responsible for several outbreaks where the evidence given by the persons affected pointed to the conclusion that the meat was apparently normal. Williams,¹² recording an outbreak caused by the consumption of pork pies contaminated with *B. paratyphosus* B¹³ (*B. aertrycke*?), states that "no person complained of the pies at the time of eating;

⁷ Quoted by Müller, M.: *Ztschr. f. Hyg. u. Infektionskr.*, 1910, 8, p. 251.

⁸ *Ztschr. f. Hyg. u. Infektionskr.*, 1896, 22, p. 53.

⁹ *Brit. Med. Jour.*, 1898, 2, p. 1456.

¹⁰ *Ibid.*, 1899, 2, p. 1367.

¹¹ *Ztschr. f. Hyg. u. Infektionskr.*, 1906, 55, p. 295.

¹² *Jour. Royal Inst. Public Health*, 1910, 18, p. 725; see also Trammsdorff, Rajchmann, and Porter, *Jour. Hyg.*, 1911, 11, p. 89.

¹³ In many of the earlier outbreaks the causative organism, if positive agglutination tests were secured with paratyphoid B serum, was not differentiated by absorption tests from the true human *B. paratyphosus* B, or *B. schottmülleri*.

indeed, the evidence went to show that they were apparently good and that there was nothing unusual in the taste." McWeeney¹⁴ makes a similar statement in his report of an outbreak traced to *B. enteritidis* beef and also records some of his own experiments in which he found that the strain of *B. enteritidis* obtained from the outbreak multiplied and spread rapidly in the beef while the meat exhibited no perceptible alteration. An extensive outbreak traced to the consumption of sausage from which *B. paratyphosus* B¹³ was obtained is reported by Federschmidt.¹⁵ The evidence collected from those affected indicated clearly that there was nothing suspicious about the taste or smell of the sausage.

The reports of outbreaks in which foods other than meat were responsible for conveying the infective organism show that the same conclusion may hold true in regard to various miscellaneous foodstuffs. An apple pudding (containing also milk, eggs, and other ingredients) contaminated with an organism quite similar to *B. paratyphosus* B was said (Vagedes¹⁶) to present no evidence of contamination. A sour taste noted by some of the affected persons was believed to be due to the apples. One of the most extensive outbreaks occurring in this country was traced to contaminated pie dough by Bernstein and Fish¹⁷ who report that there was "no unusual taste or odor to the pies to excite suspicion." A recent outbreak in France followed the use of cream containing *B. paratyphosus* B¹³ as an ingredient in preparing cakes (Lesné, Violle, and Langle¹⁸). Here again the evidence is similar to that previously reported. Sewell, Smith and Priestley¹⁹ give an account of an outbreak in an English army hospital attributed to the contamination of unsweetened canned milk with *B. aertrycke*, mutton strain. They state that cultures of this organism isolated from feces, vomitus, and postmortem were found to be able "to grow freely in the particular brand of milk used, at room temperature, and without changing the appearance or smell of the milk."

On the other hand, there are several observations in the literature which are contradictory, at least in part, to the foregoing statements. Kerr and Hutchens²⁰ while studying an outbreak traced to *B. enteritidis* in raw market milk, found that a number of the affected persons volunteered the information that the particular lot of milk under sus-

¹⁴ Brit. Med. Jour., 1909, No. 2524, p. 1171.

¹⁵ München. med. Wchnschr., 1920, 67, p. 814.

¹⁶ Klin. Jahrb., 1905, 14, p. 517.

¹⁷ Jour. Am. Med. Assn., 1916, 66, p. 167.

¹⁸ Presse méd., 1920, No. 74, p. 725.

¹⁹ Jour. Roy. Army Med. Corps, 1920, 34, p. 510.

²⁰ Proc. Royal Soc. Med., 114 (Epidem. Section), 7, p. 171.

picion had had a peculiar taste. Rosenau and Weiss²¹ quite recently have reported an outbreak following the consumption of bread pudding from which *B. enteritidis* was isolated. It is interesting to note that 4 of the 18 persons affected said that "the bread pudding had a peculiar taste" and one of them refused to eat it after the first spoonful. Since there is no evidence in either of the cases mentioned in the foregoing that the peculiarity of taste was due especially to the presence of *B. enteritidis*, rather than to the development of any one member of an undoubtedly large miscellaneous flora, these two instances, while exceedingly interesting, do not seriously detract from the general conclusion reached on the review of the cases cited.

In addition to the foregoing observations made in connection with "food poisoning" outbreaks, several additional statements appearing in the literature should be included here. In 1898, Dunham²² in a discussion of "meat poisoning," stated that "a mere naked eye inspection of the meat will not exclude danger, since meat infected with *B. enteritidis* may have quite a good appearance." Cathcart,²³ discussing the toxin of the Gaertner bacillus, remarks that food contaminated with this organism is "quite without smell." In the course of a study of the souring of beef, Bunyea²⁴ inoculated pieces of sterile normal beef with several members of the paratyphoid group. After 4 days' incubation at 37 C. no perceptible odor was produced.

These various reports, then, corroborate the conclusion reached as a result of the present work, and it appears that extensive multiplication of the paratyphoid-enteritidis group of organisms may take place in most foodstuffs with little or no alteration in either appearance, odor, or, perhaps, taste. As a result there will be little or no gross physical evidence to warn either the person preparing the meal or the consumer of possible danger.

This is decidedly in contrast to our opinion of most foodstuffs contaminated with *botulinus*. Many of the recent reports of outbreaks of botulism state that the infected food was noted to be abnormal with respect to either the odor, appearance, or swelled condition of the container. Experimental inoculation of several foodstuffs in the laboratory has verified this. Since most of the *botulinus* foodstuffs were preserved either in tins or glass containers, it is evident that there was an accumulation of metabolic products of growth, as for instance odors or gases

²¹ Jour. Am. Med. Assn., 1921, 77, p. 1984.

²² Brit. Med. Jour., 1898, p. 1797.

²³ Jour. Hyg., 1906, 6, p. 112.

²⁴ Jour. Agri. Res., 1921, 21, p. 689.

which serve to mark the food as "spoiled." In contrast to this the foods—meat, milk, puddings, pastry, soups, etc.—which commonly have been contaminated with one or another member of the Gaertner group, are not canned. Thus, aside from fundamental differences in the metabolism of the two groups of "food poisoning" organisms, the conditions under which the food is preserved or held no doubt play an important rôle in determining the extent of gross physical evidence of spoilage, one of the important links in the chain of protection afforded the consumer. Having seen that Gaertner group organisms usually give no evidence of their presence in a foodstuff, it does not seem amiss to emphasize the necessity for thorough cooking of all foods, especially those which have been exposed to an incubation period. The instances are numerous in which these organisms, after gaining entrance to a food, have survived a period of cooking insufficient to cause the penetration of heat to the center of the mass.

SUMMARY

The aim of the present investigation has been to gain some idea of the ability of several type strains of the paratyphoid-enteritidis group to develop in miscellaneous foodstuffs, such as various vegetables, fruits, meats, and evaporated milk. The effects of different conditions, such as temperature of incubation, the hydrogen-ion concentration and the texture of the food, were considered in relation to multiplication and the ability to spread throughout the foodstuff.

All the strains of the Gaertner group multiplied readily in the liquor of several common cooked vegetables, with the exception of the highly acid sauerkraut. In the fruit juices a rapid destruction of the organisms occurred. In several meat products Gaertner group organisms exhibited a marked ability to spread from one original point of inoculation throughout the foodstuff, although this occurred only under optimum temperature conditions.

The development of the Gaertner group in foodstuffs is usually not accompanied by visible alteration or spoilage. The present observations on this point are in accord with the reports of previous outbreaks of "food poisoning" caused by this group of organisms.

THE BACTERIOLOGY OF THE SKIN LESIONS IN SMALLPOX

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Twenty cases of smallpox in a mild epidemic in Chicago in the winter 1921-22 have been studied through the cooperation of Dr. A. Hoyne of the Chicago Health Department. There were 3 severe confluent cases that terminated fatally, 6 were of a mild type with only a few discrete lesions, and the remaining 11 might be classed as of a severe discrete type because of the great number of lesions and severity of the symptoms.

The contents of the lesions, when in the vesicular and pustular stages, were used for cultures and smears. Blood cultures were available in only a few of the cases. The method of procedure was as follows: After sponging the lesion with alcohol, sterile pipets were inserted and the contents withdrawn. Smears were made of a portion and the remainder transferred to a nutrient broth containing 0.5% dextrose, and incubated under aerobic and anaerobic conditions for 24 hours. The growth was then plated by surface inoculation on sheep blood-agar plates, containing 1 part of citrated sheep blood to 10 parts of plain agar.

As shown in table 1, streptococci were obtained by cultures from the lesions in 5 of the 20 cases. Hemolytic streptococci were present in 2 and nonhemolytic streptococci in 1 of the 3 fatal cases. Staphylococcus albus was found in combination with streptococci in 2 cases and alone in 7 cases. Once a hemolytic staphylococcus was present in combination with a streptococcus. Of the 17 patients that recovered, streptococci were obtained from the lesions in 2 instances or in less than 12%. No streptococci were obtained during the vesicular stage.

Three of the strains of streptococci were of the hemolytic and 2 of the viridans type. The strains of hemolytic streptococci from the lesions, as well as the hemolytic streptococci from the throats of the corresponding patients, were gram-positive and produced a wide zone of hemolysis (3 to 5 mm. in width) on sheep blood-agar plates in 24 hours. They fermented dextrose, maltose, lactose, and salicin but did not ferment mannite inulin, dulcitate or raffinose and therefore

would be classed as *Streptococcus pyogenes* (Holman¹). The 2 viridans strains were gram-positive, bile insoluble and of the streptococcus mitis group. Rabbits were injected intravenously with 2 of the strains of hemolytic streptococci. Suspensions of a 24 hour growth on a blood-agar slant were made in normal salt solution and heated for 30 minutes at 56 C. The first injection was $\frac{1}{4}$ of a slant and the dose was doubled with each succeeding injection at intervals of 7 days. Four days after the fourth injection the serum usually gave a satisfactory agglutinin titer. Daily repeated subcultures in broth containing 1% dibasic sodium phosphate and 0.5% dextrose was the means of overcoming the tendency of the cultures to spontaneous agglutination. The antiserum for the streptococci from the lesions of case 2 agglutinated suspensions of hemolytic streptococci from the throat in acute tonsillitis as well as from smallpox lesions, while the antiserum for the streptococcus from the lesions of case 14 contained agglutinins for that strain only.

It is well known that streptococci are found in the blood in fatal cases of smallpox. In 40 necropsies, Perkins and Pay² found them in the heart blood and viscera of 95%. Their observations were made before the streptococci had been separated into hemolytic and non-hemolytic groups. Studying typical variolous lesions at all stages they found streptococci in only 4 of 30 cases while De Wael and Sugg³ found streptococci constantly in smallpox pustules.

If the rôle of streptococci is that of a secondary invader only, one would expect to find them in the later stages of the disease, after the resistance of the patient has been lowered. Thus Perkins and Pay² found them in the skin lesions only after the eighth day. Invasion may occur earlier than this, however, for Kempton and Parsons⁴ report a case of purpura variolosa in which hemolytic streptococci were obtained from the blood 36 hours after the onset of symptoms and before the appearance of the eruption. The one case of purpura variolosa in my series was not observed before the eighth day of the disease when streptococci were abundant in the lesions.

SUMMARY

Hemolytic streptococci occurred in the pustules of smallpox in 3 and nonhemolytic in 2 of 20 cases. Staphylococci were found in

¹ Jour. Med. Res., 1916, 34, p. 377.

² Jour. Med. Res., 1903, 10, p. 180.

³ Arch. Int. de Pharm. et de Therap., 1903, 12, p. 105.

⁴ Arch. Int. Med., 1920, 26, p. 594.

seven cases. Death took place in 3 cases and streptococci were found in the skin lesions of all 3. The agglutinin test indicated that the hemolytic streptococci found in the skin lesions belonged to different groups.

TABLE 1
SUMMARY OF RESULTS IN TWENTY CASES OF SMALLPOX

Cases	Type of Disease	Day of Disease	Lesions Cultured	Results	
				Smears	Cultures
1	Mild, discrete.....	7	Vesicle.....	0	0
		10	Pustule.....	0	0
		17	Dried pustule.....	0	0
2	Confluent, fatal....	8	Pustule.....	Streptococci	Hemolytic streptococci
		11	Pustule.....	Streptococci	Hemolytic streptococci
		8	Pustule.....	Streptococci and staphylococci	Hemolytic streptococci and staphylococci
4	Severe, confluent....	30	Secondary abscess of foot	0	0
5	Mild, discrete.....	10	Pustule.....	0	0
		15	Dried pustule.....	0	0
6	Severe, discrete.....	8	Vesicle.....	0	0
		10	Pustule.....	Streptococci	Hemolytic streptococci and staphylococci albus
7	Severe, discrete.....	8	Vesicle.....	0	0
		10	Pustule.....	0	0
		12	Pustule.....	Staphylococci	Staphylococcus albus and diphtheroid bacilli
8	Severe, discrete.....	8	Late vesicle.....	0	0
		10	Pustule.....	0	0
		12	Pustule.....	0	Staphylococcus albus
9	Mild, discrete.....	6	Vesicle.....	0	0
		9	Pustule.....	0	0
10	Severe, semiconfluent	7	Vesicle.....	0	0
		9	Pustule.....	0	Staphylococci
11	Severe, discrete.....	6	Vesicle.....	0	Staphylococcus albus
		8	Pustule.....	Staphylococci	Staphylococcus albus
		8	Pustule.....	0	0
12	Mild, discrete.....	8	Pustule.....	0	Staphylococcus aureus
13	Mild, discrete.....	6	Vesicle.....	0	Staphylococcus albus
		8	Pustule.....	Staphylococci	Staphylococcus albus
14	Very severe, confluent of face, discrete of body	7	Vesicle.....	0	0
		9	Pustule.....	Streptococci	Hemolytic streptococci
15	Mild, discrete.....	9	Blood culture.....	—	0
		7	Vesicle.....	0	0
		9	Pustule.....	Staphylococci	Staphylococcus albus
16	Moderate, discrete..	6	Vesicle.....	0	0
		9	Pustule.....	0	0
17	Moderate, discrete..	7	Pustule.....	0	0
		9	Pustule.....	0	0
18	Very mild.....	8	Pustule.....	0	0
		10	Pustule.....	0	0
19	Severe, discrete.....	7	Vesicle.....	0	0
		9	Pustule.....	0	Staphylococcus albus
20	Hemorrhagic pustulous, fatal	8	Pustule.....	Streptococci and staphylococci	Streptococcus viridans and hemolytic staphylococci

CLOSTRIDIUM BOTULINUM

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After the relation of *Clostridium botulinum* to food poisoning had been established, the attention of bacteriologists was directed toward a better understanding of the characteristics of the causal organism. Practically all of our information rested on data in the publications of van Ermengem. Many of the observations do not seem to hold for the strains which have been isolated in America. The object of our work was to seek information concerning the reaction to heat and the natural habitat of the organism. It is one of the many which will have to be carried out before we can understand *Clostridium botulinum* as well as some other pathogenic anaerobes.

I. RESISTANCE OF SPORES OF CLOSTRIDIUM BOTULINUM TO DRY HEAT

Since *Clostridium botulinum* forms spores which are resistant to moist heat, it was thought necessary to determine whether or not the dry heat methods of sterilization are effective in destroying the spores. Tests were made on five of the cultures isolated from various sources.

A rich spore culture in brain medium was used. The culture tubes were closed with sterile corks and put on the shaking machine for about 5 minutes. This was done in order to secure as even distribution of spores throughout the medium as possible. Sterile tubes plugged with cotton were swabbed out with these cultures. By this method approximately the same number of spores was present in each tube. These tubes were inverted into large beakers, the bottoms of which were covered by several thicknesses of sterile filter paper. The plugs taken from the tubes were put into a tin receiving can which was covered and which had been sterilized for several hours. After the moisture had drained from the tubes, the sterile plugs were replaced and the tubes were then ready to be heated. A gas oven of the Lautenschläger type which is used for sterilizing glass ware in the laboratory was used. Some difficulty was experienced at first in keeping the temperature constant since a variation in gas pressure is inevitable in a large labora-

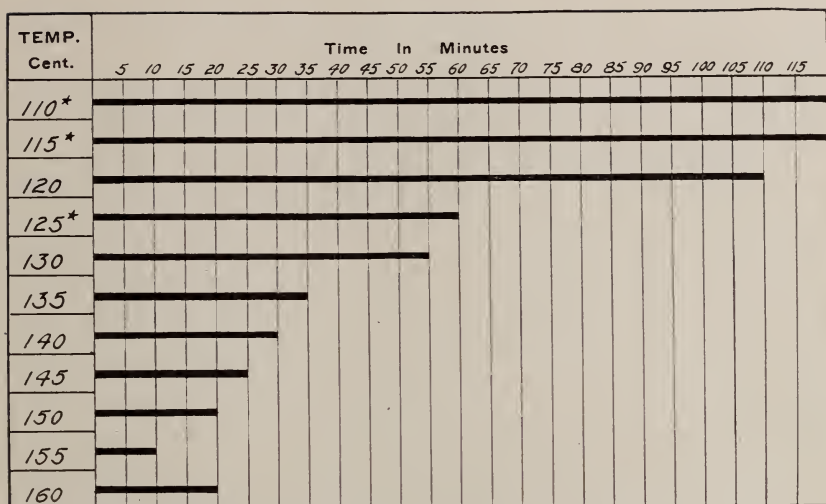


Chart 1.—Culture 7-p-1. All spores were 10 days old. The asterisks indicate that the spores survived the period of observation.

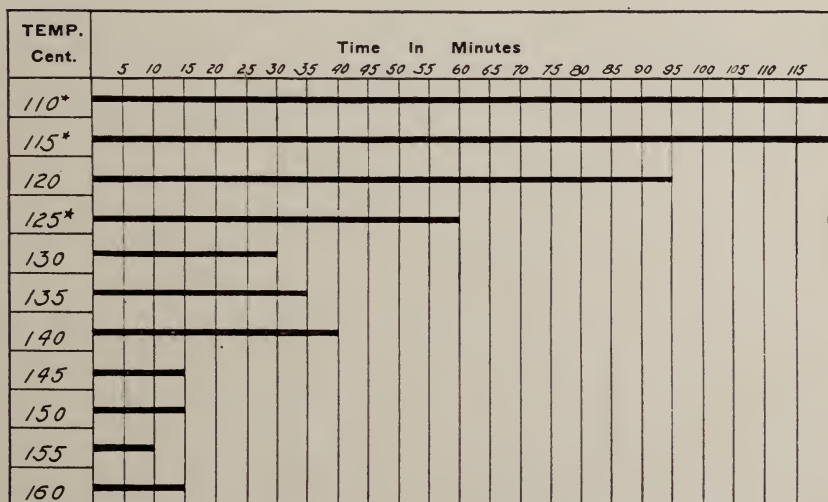


Chart 2.—Culture 2-9. All spores were 10 days old. The asterisks indicate that the spores survived the periods of observation.

tory in which many burners are in use. As the study progressed, however, it became easier to maintain a given temperature within narrow limits.

In making a test the oven was first regulated to the temperature at which the test was to be carried out. At the lower temperature the oven could not be regulated more closely than within 2 to 3 degrees of the temperature desired. Tubes were removed at various intervals. They were allowed to cool and sterile dextrose broth (9 cc to a tube) was added, after which they were sealed with parawax and incubated at 37 C. for a period sufficient to allow the development of viable spores. Viable spores were indicated by turbidity of the culture medium, formation of gas which sometimes forced the parawax upward in the tube, and by feeding tests for the presence of toxin. It was frequently noticed that growth was delayed in some of the tubes, probably due to injured spores becoming slowly viable.

The results of exposure of botulinus spores to dry heat are indicated in the charts presented herewith. It will be seen that, as would be expected, the spores survived longer at the lower temperatures. The charts for cultures 4-5, 2-9, and 7-p-1 show a progressive decrease in resistance under the conditions which obtained in the experiments. Cultures G7870, and 3p-9 show more irregular reactions, especially when compared with the other 3 cultures. For all the tests homogeneous spore suspension of the same ages were used to insure comparable data. Two exceptions were made in cultures No. 7870 and 3p-9 for the tests made at 135 C. The ages of the spores are given under the charts.

II. DISTRIBUTION IN SOIL AND FECES

The distribution of the spores of *Clostridium botulinum* is not well understood. Several investigations have been reported but more will be necessary before our information is firmly established.

Burke¹ isolated *Clostridium botulinum* from various sources. Cultures were made in double strength beef infusion broth with 2% glucose. Oil stratification was used to insure anaerobiosis. Other factors were also considered. At the end of the incubation period the culture was filtered and 1 cc of the filtrate was injected, subcutaneously, into a guinea-pig, 235 cultures were made from samples of various materials collected in 5 localities in central California, 50 or more miles distant from each other. Seven cultures of *Clostridium botulinum* were found. *Clostridium botulinum* was isolated from the following: bruised moldy cherries, bird pecked cherries, pole bean leaf covered with spots or droppings of insects or small animals, spiders from bush bean plants, bush beans, some of which were slightly scarred, picked over, washed and packed in

¹ Jour. Bacteriol., 1919, 4, p. 541.

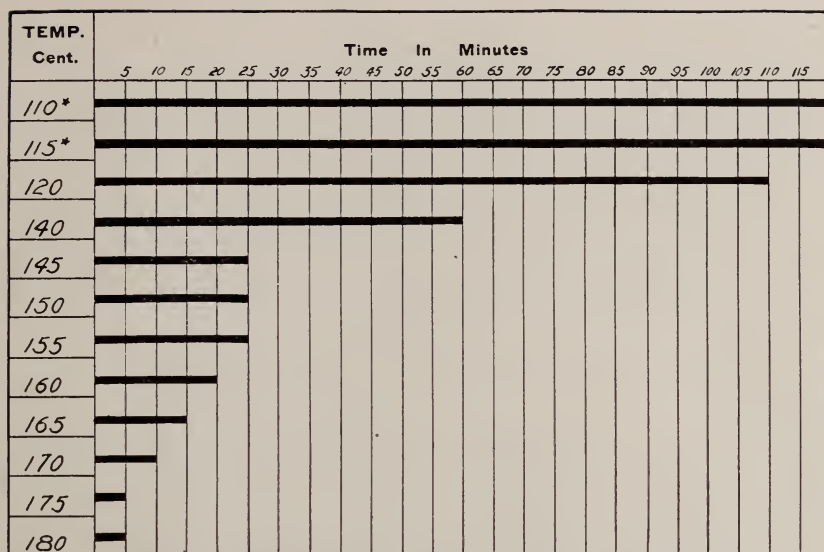


Chart 3.—Culture 4-5. All spores were between 10 and 20 days old. The asterisks indicate that the spores survived the period of heating.

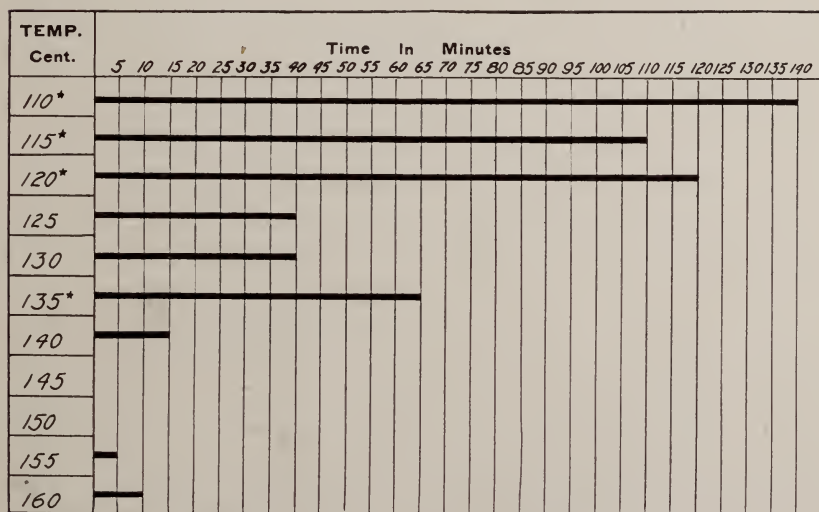


Chart 4.—Culture 3 p-9. All spores were 90 days old with the exception of those used at 135 C. which were 10 days old. The increased resistance of young spores compared with old spores is brought out. The asterisks indicate that the organisms survived the period of heating.

clean jars for canning; manure from a large hog which had recovered from botulism 3 months before the sample was taken and from discolored moldy hay from an outdoor stack.

Meyer and Geiger² believe that the spores may be widely distributed in nature in certain localities. Thus, it may also be found on fruits, vegetables, etc., raised in these localities. They suggest the possibility of certain animals being spore carriers. This opinion seems to be borne out by the present study.

Thirty-three samples of soils were collected from numerous places around Urbana: gardens, pastures, hog lots, corn fields, oat fields, etc. Twenty-two samples, collected from various parts of the state, were secured from Prof. R. Graham of the Department of Animal Pathology.

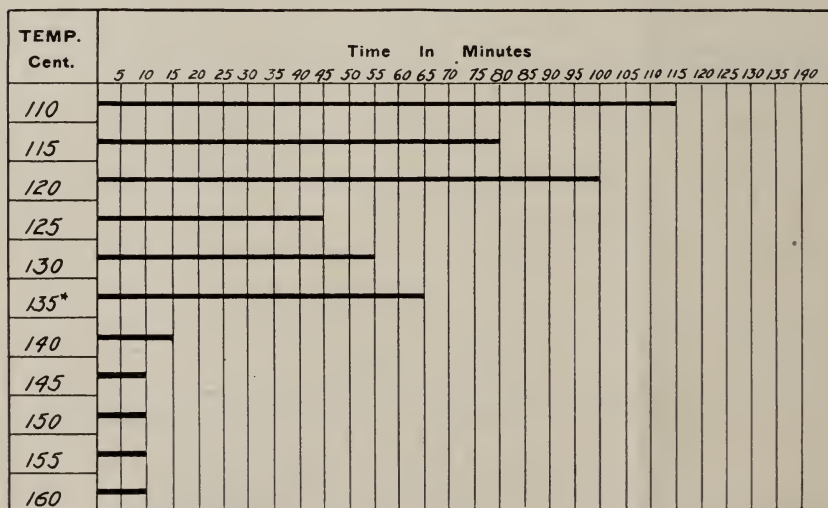


Chart 5.—Culture G 7870. All spores used were 90 days old with the exception of those used at 135 C. These spores were 10 days old. The increased resistance of these young spores is clearly brought out. The asterisk indicates that the spores survived the period of heating employed.

The technic first used was improved and modified slightly as the studies progressed. The samples of soil were put into sterile tubes and diluted with an equal volume of distilled water, or physiologic salt solution. They were then shaken on a shaking machine for about 20 minutes. This gave a homogeneous suspension. They were allowed to settle and the supernatant liquid was transferred to sterile tubes. The tubes were stoppered and heated in a water bath for 30 minutes at 80 C. Open tubes were used in the early part of the experiment.

² Pub. Health Repts., 1921, 36, p. 4.

TABLE 1
DISTRIBUTION OF CLOSTRIDIUM BOTULINUM IN SOIL AND FECES

Sample	Source	Toxin	Botulinus as Confirmed by Anti- toxin Type
4	Soil from old hog lot not in use at present time.....	+	B
5	Soil from dry hog lot.....	+	B
6	Soil from vegetable garden.....	+	B
7	Soil from city garden.....	+	B
8	Soil from garden.....	+	B
9	Soil from land that has not been manured within past 5 years.....	—	—
10	Soil from land which has received manure.....	—	—
11	Soil from land which has received manure.....	—	—
12	Soil from land that has not been manured within the past 5 years.....	—	—
16	Soil from experimental plot.....	—	—
17	Soil from vegetable garden.....	—	—
18	Garden soil.....	+	+
19	Garden soil.....	—	—
20	Soil from city garden.....	—	—
21	Soil from garden (new ground).....	—	—
22, 23, 24	Garden soil.....	—	—
25	Soil from corn field.....	—	—
26	Soil from corn patch; manured land.....	—	—
27	Soil from land next to railroad; sown to oats.....	—	—
28	Soil from pasture.....	—	—
29	Soil from corn breeding plots; land formerly in cat- tle feeding lots.....	—	—
30	Soil from oat field.....	—	—
31	Soil from corn field.....	—	—
32	Soil from oat field.....	+	+
33	Soil from corn field.....	—	—
34	Soil from pasture.....	—	—
35	Soil from pea field, Northern Illinois.....	—	—
36	Soil from pea field, Northern Illinois.....	—	—
37	Soil from pea field, Northern Illinois.....	—	—
38	Soil from pea field, Northern Illinois.....	—	—
39	Soil from pea field, Northern Illinois.....	+	+
40	Garden soil, land manured, La Junta, Colo.	—	—
41	Garden soil, Everett, Wash.	—	—
42	Garden soil, Warsaw, N. Y.	—	—
43	Garden soil, Germania, Pa.	—	—
44	Garden soil, Portland, Ore.	—	—
45	Garden soil, Elgin, Ill.	—	—
46	Garden soil, Portland, Me.	—	—
47	Garden soil, New London, Conn.	—	—
48	Garden soil, Springfield, Mass.	—	—
49	Garden soil, Detroit, Mich.	—	—
50	Garden soil, Richmond, Va.	—	—
51	Garden soil, Winnebago, Ill.	—	—
52	Garden soil, Salem, Ore.	—	—
53	Garden soil, Halstead, Kan.	—	—
54	Garden soil, Vermont.....	—	—
55	Garden soil, Chepachet, R. I.	—	—
56	Garden soil, Oakland, Calif.	+	B
57	Garden soil, New Orleans, La.	—	—
G 570, 687, 1133, 1313, 1433, 1505, 2435, 2483, 4073, 4156, 4445, 5324, 6713, 7399, 7414, 7480, 7585, 7765, 8305 7870 G 7855	From Dr. Graham, samples from all over state..... From Dr. Graham..... From Dr. Graham.....	— + +	— B +

Various portions of the liquid were inoculated into brain medium prepared after the method of Dickson and Burke.³ Varying numbers of drops of the liquid were also plated out in dextrose agar, and anaerobic conditions were obtained according to Dick's method. These anaerobic plates were then incubated for 48 hours or longer at 37 C. Sometimes this was combined with incubation at room temperature. From these plates inoculations were made from different suspicious colonies into 36 tubes of sheep brain medium. This sheep brain medium was found to be satisfactory since it seemed to contain the types of food materials most easily assimilated by *Clostridium botulinum*.

The tubes of brain medium were sealed with parawax and incubated at 37 C. for about 10 days. They were then examined and where gas formation was found accompanied by blackening of the medium with

TABLE 2
CLOSTRIDIUM BOTULINUM IN FECES AND SEWAGE

Sample	Source	Toxin	Botulinus as Confirmed by Anti- toxin Type
1	Feces from hog in apparently good health.....	+	B
2	Feces from hog in apparently good health.....	+	B
3	Feces from hog in apparently good health.....	+	B
13, 14, 15	Feces from test cows.....	—	—
58	Champaign, Ill., sewage.....	+	B

a characteristic putrid odor, they were regarded as suspicious and were held for animal inoculation. One c c of this brain medium was fed to guinea-pigs from a pipet. With strong toxins the animals showed symptoms in 5 or 6 hours and died in 10 hours. With the weaker toxins, however, death was delayed for several days. We noticed a great difference in the strength of toxins formed by these strains isolated from soil.

If toxin was found to be present as evidenced by the death of a guinea-pig with the usual symptoms of botulism in these animals, the type of the culture was determined by the use of types A and B anti-toxin. Guinea-pigs dying with the usual symptoms of botulism were examined and cultures made from various organs. No difficulty was experienced in finding the organism in the brain tissues of animals showing latent death in many of these necropsies. Orr⁴ has recently reported the isolation of the organism "from the organs of animals

³ Jour. Amer. Med. Assn., 1918, 71, p. 518.

⁴ Jour. Infect. Dis., 1922, 30, p. 118.

which have died following the administration of toxin-free spores either subcutaneously or by the mouth." When the organism was isolated from the brain of guinea-pigs dying from oral administration of sheep-brain medium culture (spores and toxin) it was again fed to guinea-pigs for the purpose of fulfilling Koch's postulates. No complete cultural studies were carried out. Strains of cultures revealed the usual clostridium rods.

The results of this investigation show that of the 73 samples of soil examined 11 showed the presence of an organism producing the usual symptoms of botulism in guinea-pigs. Seven of these cultures produced sufficiently strong toxins to cause the death of guinea-pigs in from 12 to 15 hours. All of these were found to be type B organisms. Four others, 18, 32, 39, and G7855 contained organisms producing a weak toxin which caused a delayed death in guinea-pigs. The symptoms, however, were those of botulism. These strains were not typed.

EXPERIMENTAL STUDY OF OCCURRENCE OF CLOSTRIDIUM BOTULINUM IN FECES AND SEWAGE

Six samples of animal feces and one sample of sewage were tested for the presence of *Clostridium botulinum*. Three specimens were from hogs and 3 from dairy cattle. All samples were fresh when taken. The samples were treated in exactly the same way as the soil samples. From table 3 it will be seen that the 3 samples from hogs were positive. Such data have an interesting bearing on the statement of van Ermengem that the organism might be a normal inhabitant of the intestinal tract of the hog. The 3 specimens from cows gave negative results. A series of specimens of human stools is in progress of examination.

The one specimen of raw sewage was found to contain the organism. This strain produced a powerful toxin which caused typical symptoms in guinea-pigs. Polyvalent antitoxin protected guinea-pigs against this antitoxin, but the use of homologous antitoxin showed it to be type B.

SUMMARY AND CONCLUSIONS

The different strains of *Clostridium botulinum* in this investigation showed different resistance to dry heat. This was probably due to inherent characteristics and in a large measure to the age of the cultures used. At 110 C. the time of survival averaged beyond 120 minutes. At 140 C. the variation was between 60 minutes and 15 minutes, a rather wide variation. At higher temperatures of 160 C. and 180 C.

the times of survival were short, between 5 and 15 minutes. The modern methods of dry heat sterilization seem, then, to be adequate for sterilizing apparatus which has been used for cultivating *Clostridium botulinum*. Young spores of *Clostridium botulinum* are more resistant to dry heat than old ones.

Clostridium botulinum like other pathogenic anaerobes is commonly present in nature. In this investigation 11 of 73 of the samples of soil contained it.

Three specimens of hog feces contained it, but it was not isolated from 3 specimens of cow feces. It was isolated from one sample of sewage.

Clostridium botulinum is probably a common saprophyte widespread in nature. The results of this investigation do not conflict with those of Meyer and Geiger who propose a regional distribution of the organism. It may be that it is more common in recently manured soils.

Further investigation must be carried out before a better understanding of the characteristics of *Clostridium botulinum* is attained. The occurrence of the organism in stools of healthy individuals is one phase of the subject which is now being investigated.

EXPERIMENTAL ERYSIPELAS

STUDIES IN STREPTOCOCCUS INFECTION AND IMMUNITY. IV *

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Erysipelas was probably the first recognized of those human diseases which we now know to be due to the streptococcus. Its striking clinical appearance made it recognizable as an entity even in ancient times. Erysipelas is a disease of much interest as the first of the streptococcus infections from which the causative organism was isolated. It is a disease the instances of which are apparently diminishing, owing largely to the introduction of aseptic and antiseptic treatment of wounds.

The essential facts concerning clinical erysipelas may be briefly outlined as follows:¹

The forms of erysipelas have at times been classified as idiopathic and traumatic. From the present conception, however, this point of differentiation is scarcely valid, since trauma must be present even in the so-called idiopathic forms. Erysipelas tends to occur in families, and is present more frequently in adults and in old age. There are said to be more cases among females than among males, although it is more fatal in the latter sex. The occurrence of erysipelas in children, though not so notable, is marked by great severity. The mortality in general is given as from 3-10%.

Erysipelas occurs most frequently on the face or back, and in infants about the umbilicus. The inoculation, which occurs through slight abrasion or as a spreading process from some previously infected wound, is followed by a short period of incubation, of from 15 to 60 hours. The characteristic lesion of erysipelas is an infection of the superficial layers of skin and spreads by extension. In general it takes the form of a sharply demarcated, red, hot, indurated area, which spreads rather rapidly with a characteristically raised, glistening margin, leaving behind, as the process extends, a less inflamed yellowish or

Received for publication April 18, 1922.

* Preceding studies in this series are:

Gay, F. P., and Stone, R. L.: *Jour. Infect. Dis.*, 1920, 26, p. 265.

Gay, F. P., and Morrison, L. F.: *Ibid.*, 1921, 28, p. 1.

Gay, F. P., and Rhodes, B.: *Ibid.*, 29, p. 217.

Of correlative interest are the following articles by our associates:

Cook, M. W.; Mix, V., and Culvyhouse, E. O.: *Ibid.*, 1921, 28, p. 93.

Foster, L. F., and Randall, S. B.: *Jour. Bacteriol.*, 1921, 6, p. 143.

Foster, L. F.: *Ibid.*, p. 161.

Foster, L. F.: *Ibid.*, p. 211.

Aided by grants from the Research Board of the University of California and from the Council on Pharmacy and Chemistry of the American Medical Association.

¹ Lambert, A. V. S.: *Nelson System of Med.*, 1920, 1, p. 446. Tileston, W.: *Oxford Med.*, 1921, 4, p. 887. Anders, J. M.: *Osler's Modern Med.*, 1907, 2, p. 525. Kolle, W., and Hetsch, H.: *Die exper. Bakteriologie und die Infektionskrankheiten*, 1911, 1, p. 397. Jochmann: *Lehrbuch der inf. Krankheiten*, 1914, p. 521. Ker, C. B.: *Oxford Pub.*, 1920, p. 465. Sørensen, E.: *Ztschr. f. Hyg. u. Infektionskr.*, 1908, 62, p. 363.

brownish center. This spreading infection is accompanied by a fever of considerably intensity, to 104 F. or even higher, which is continuous or remittent in form and usually ends by crisis. Erysipelas is not ordinarily a painful infection, but it is accompanied by a sensation of burning. Blebs occur frequently on the surface; suppuration, though often present, is not a characteristic feature.

A more minute examination of the lesion itself shows that the streptococcus, which is the cause of the disease, is found in greatest numbers in the advancing indurated margin. The organisms are seen filling the lymphatic spaces of the corium, which explains the method of the spread of the infection. The advancing margin of infection is not characterized by the occurrence of any considerable number of polymorphonuclear leukocytes, as is usual in the more pyogenic types of streptococcus infection, but by the presence of mononuclear cells (MacCallum²). According to Unna,³ the streptococcus of erysipelas is negatively chemotactic for leukocytes that bear the oxygen necessary for its growth. It, therefore, must seek those portions of the skin in the lymphatic spaces and connective tissue that contain most oxygen, which explains its characteristic localization. Dead streptococci, however, fall prey to the polymorphonuclears, which accounts for the accumulation of these cells in the older regions of the process and the formation of a certain amount, though never of a predominating quantity, of pus. The former contention of Fehleisen⁴ that true erysipelas must by definition be a nonsuppurative process would, we believe, be no longer tenable.

Erysipelas is usually self-limiting and does not ordinarily produce complications in the form of pyogenic foci or septicemia. The process lasts in uncomplicated instances for from 4 to 14 days.

Relapses or exacerbations of erysipelas are said to occur in about 10% of the cases, that is to say, relatively frequently. These relapses should be differentiated from the recurrences of the process; which have been regarded as one of its most characteristic features. It has been recognized that there exists a wide individual variation in susceptibility to erysipelas, as is evidenced not only by the nonoccurrence of the process in most persons, but particularly by the repeated occurrence in certain hypersusceptible persons. Various estimates have shown that adults suffering from erysipelas give a history of previous attacks in from 9 (Anders) to 20% (Tileston) of cases. As many as 40 attacks have been reported in a single person. This tendency to recurrence of the disease is one of the arguments that has been used to prove the nonexistence of any permanent active immunity against streptococcus infections. There are, however, other data, particularly of an experimental nature, in man as well as in animals, which seem to show equally clearly that recovery from one attack is followed by at least a transitory protection. It is evident that some form of increased resistance must occur or there would be no self-limitation of the process (Kolle and Hetsch⁵). That recovery is not due to the dying out of the streptococci, but to a real though temporary increased resistance, is evidenced by the fact that the organisms have been found for considerable periods after recovery in the scales of the subsiding lesion (Nobel and Zilcher⁶). This is further indicated in the observations of Levaditi,⁷ who found that healing streptococcus wounds have the property of destroying the particular streptococci that had been present in them. This matter of temporary, and perhaps local, acquired

² Text Book of Pathology, 1920, p. 521.

³ Deutsch. med. Wchnschr., 1921, 47, p. 1349.

⁴ Fehleisen, F., 1883.

⁵ Med. Klin., 1918, 14, p. 491.

⁶ Compt. rend. Soc. Biol., 1918, 81, p. 1059.

immunity is one that interests us primarily in this article and can be more profitably discussed in connection with the experimental production of erysipelas in rabbits.

Experimental erysipelas was probably first produced by Orth⁷ in 1873, who, before the knowledge of the causative organism in this disease, produced a spreading and fatal abscess by subcutaneous inoculation on the back of rabbits of the contents from a human erysipelatous bleb. It is quite possible also that he actually succeeded in growing pure cultures of the "microspheres," which he found associated with both the natural and experimental lesion. In 1881 Fehleisen⁸ began his studies on the histologic findings in cases of erysipelas, and in 1883 published his completed work, which included the growth in pure culture of the streptococcus constantly present in this lesion, to which he gave the name of *Streptococcus erysipelatis*. This streptococcus was not only present in the lymph spaces of the corium, particularly about the edges of the lesion, but after growth in blood serum and gelatine was capable of reproducing the disease when inoculated subcutaneously or by scarification on the ear in 9 rabbits. An incubation period of 3 or 4 days was followed by a rise in temperature, a sharply defined reddening, which extended to the root of the ear or over the head and neck. Fehleisen also reproduced the disease with this culture in several human beings suffering from malignant tumors, in some of whom a temporary retrogression of the tumor followed. There was likewise some evidence in the work of Fehleisen, as he himself appreciates from the study of these human cases, that a transitory immunity to reinfection with erysipelas exists, since second inoculation in certain of the originally positive cases resulted in failure.

The immediate sequel to this epoch-making study of Fehleisen was a discussion as to the specificity of the particular organism which he and subsequently others were able to isolate from this disease. In the following year, 1884, Rosenbach⁸ isolated a streptococcus from purulent processes, to which he gave the name of *Streptococcus pyogenes*. Although at first an attempt was made to differentiate between these two varieties of streptococcus, it soon became evident that it could not be done, at least on the basis of their individual pathogenic effect on animals. A fundamental misconception as to comparative pathogenicity became firmly imbedded in the minds of the bacteriologists of this era in their efforts to fulfill too literally that particular postulate of Koch which prescribes that an organism to be proved to be the cause of any given disease in man, must, after isolation in pure culture, be capable of reproducing the same disease in a laboratory animal. We now know that such reproduction of disease is often impossible, particularly in the most characteristically human maladies. Attempts are still made to judge the original pathogenic significance of organisms isolated from human beings by measuring their lethal effects in animals. It is characteristically true, however, that the streptococcus usually shows the directly opposite results when the pathogenic effects of a given strain are measured in man and in rabbits; human pathogenic strains are relatively nonpathogenic for rabbits, and the reverse is often the case. At all events, in the case of erysipelas, although it so happens that a reproduction of the human disease in rabbits is possible, this fact by no means serves to prove that the organism originally isolated from human cases is capable of producing that disease and no other, either in man or animals. It was soon shown by Biondi,⁹ Guarineri,¹⁰ Petruschky¹¹ and many others that streptococci isolated from septicemia and various pyogenic

⁷ Arch. f. Exper. Path., 1873, 1, p. 81.

⁸ Wiesbaden, 1884.

⁹ Deutsch. med. Wehnschr., 1886, N.S. p. 132.

¹⁰ Baumgartens Jahresbericht, 1887, 3, p. 32.

¹¹ Ztschr. f. Hyg. u. Infektionskr., 1896, 23, p. 142.

processes in man will all produce erysipelas in the rabbit ear equally well. It was further shown that erysipelas is not wholly etiologically specific in man. From the early work of Bonome¹² and others it has been shown that other bacteria, such as staphylococci and colon bacilli and what are now recognized to be other varieties of streptococcus, may also produce erysipelas. It should further have been evident that erysipelas is not produced by a streptococcus endowed with some specific action when we consider that the disease occurs following and by direct infection from streptococcus wounds, and conversely that erysipelas may be followed by complications in the nature of septicemia and localized abscesses. The idea of a specific localizing affinity for any given organism has, however, by no means been abandoned, as is evidenced by what is generally regarded as a misinterpretation of experimental possibilities in the recent work of Rosenow.¹³

Having determined, then, that the streptococcus from erysipelas is from the standpoint of pathogenicity not endowed with unique and unchangeable specificity, it became evident from the subsequent experimental work in rabbits that these erysipelas strains differ in the results they produce in accordance with their particular and temporary pathogenicity for rabbits. Most of the observers, for example, who immediately followed Fehleisen,⁴ notably Hajeck,¹⁴ Denuce,¹⁵ and Metchnikoff,¹⁶ produced more discrete and purulent lesions with the strains of the cocci that they had isolated. In other words, they were simply dealing with strains less pathogenic for rabbits. In other hands streptococcus cultures became so pathogenic after passage through animals as to produce septicemia and death instead of erysipelas, unless minimal doses were employed.

With the gradual differentiation of streptococci from various sources by means of their biologic reactions, it soon became clear that the streptococcus which is found in nearly every case of erysipelas belongs to the group we now classify as *Streptococcus pyogenes* in its modern acceptance as presented by Holman;¹⁷ that is to say, it is a hemolytic variety, which ferments lactose and salicin but not mannite. That this is the variety found almost without exception in erysipelas is amply corroborated by the work of Holman himself and also from the observations of Schottmueller,¹⁸ Lorey,¹⁹ Lyall,²⁰ Topley and Weir,²¹ and others. Recently it has at least been suggested in the work of Howell,²² and particularly by Tunnickliff,²³ that the particular form of *Streptococcus pyogenes* that occurs in erysipelas may present certain immunologic characteristics which would separate it from other varieties of *Streptococcus pyogenes* that occur in other diseases. This further differentiation, if true, is not of necessity consonant with the idea of specific localization that we have just mentioned and with the certainly erroneous idea of a common degree of pathogenicity between animal species. At all events, we may regard it as settled that *Streptococcus pyogenes* in its most recent sense is the cause of nearly every case of erysipelas.

Further consideration of the experimental disease as produced in rabbits leads, we believe, to certain interesting conclusions on the nature of erysipelas.

¹² Baumgartens Jahresbericht, 1886, 2, p. 15.

¹³ Jour. Am. Med. Assn., 1915, 65, p. 1687.

¹⁴ Deutsch. med. Wchnschr., 1886, No. 47.

¹⁵ Thèse de Bordeaux, 1885.

¹⁶ Virchows Archiv, 1887, 107, p. 209.

¹⁷ Jour. Med. Research, 1916, 34, p. 377.

¹⁸ München. med. Wchnschr., 1903, 50, p. 849.

¹⁹ Ztschr. Hyg. u. Infektionskr., 1909, 63, p. 135.

²⁰ Jour. Med. Research, 1914, 30, p. 487.

²¹ Jour. Path. and Bacteriol., 1921, 24, p. 333.

²² Jour. Infect. Dis., 1918, 22, p. 230.

²³ Jour. Am. Med. Assn., 1920, 75, p. 1339.

particularly in reference to the existence of transitory immunity and also as bearing on the more general problem of local, as opposed to a general, immunity.

We have already referred to the original observations of Fehleisen,⁴ who found that he could produce human erysipelas by means of pure cultures of his streptococcus in 6 out of 7 cases of malignant tumor in which he practiced inoculation. On reinjecting 2 of these 6 positive cases (cases 3 and 5) from 3 to 4 weeks subsequently, no erysipelas followed, and yet in the second of these cases the first inoculation had not only been positive, but had followed a spontaneous erysipelas which occurred about 10 months previously. These observations, limited as they were, certainly strongly suggested a transitory immunity and were so accepted by Fehleisen and later by Metchnikoff.¹⁶ They were, however, not so regarded as indicative of active immunity by Koch and Petruschky²⁴ in the light of their own experiments. The first experimental study of this condition of streptococcus immunity was, we believe, made by Meierowitsch²⁵ in 1887, who found that healed erysipelas in rabbits protects against new inoculation for a period of from 1 to 2 months. Similar observations were made by Roger²⁶ a few years later, and he makes the definite statement that not only is the inoculated ear protected, but likewise the other ear. These studies were followed by similar work by Gromakowsky,²⁷ who further makes the interesting observation that rabbits that have become insusceptible to superficial skin inoculation do not always withstand a fatal intraperitoneal dose. Denys and Leclef²⁸ produced erysipelas with very small amounts of a broth culture of some of the strains they employed and found recovery was followed by little or no reaction on second inoculation. A subsequent piece of work by Cobbett and Melsome²⁹ is interesting in this connection. They failed to obtain erysipelas on reinoculation in the same ear during a period roughly of from 22 to 100 days following the first successful inoculation. They also found that there is some resistance when the second inoculation is given in the other ear, although it is not so marked as when given in the first ear. They interpreted this partial protection of the other ear as due to a condition of general immunity as against the purely local immunity present in the first ear. A second inoculation in the protected ear causes a transitory and limited infection, which is accompanied by an exaggerated leukocytic response. In the second article of Cobbett and Melsome³⁰ an attempt is made to estimate the factors concerned in this local immunity. Roger³¹ had previously shown that successful experimental erysipelas depends rather closely on the amount of blood supplied to the part as influenced by experiments which affect the nervous control of the blood vessels. Cobbett and Melsome's work likewise indicates that a nonspecific inflammation such as that produced by mustard oil will prevent successful inoculation.

It is with these observations in mind that we have been interested in studying experimental streptococcus erysipelas more attentively. The question arises as to the actuality and duration of an acquired active immunity to this process, and further interesting relations have been suggested by this work as to the possible existence of a local immunity. The general question of a local immunity as contrasted with general immunity has been raised at intervals through the

²⁴ Ztschr. f. Hyg. u. Infektionskr., 1896, 23, pp. 267 and 474.

²⁵ Centralbl. f. Bakteriöl., Ref., 1888, 3, p. 406.

²⁶ Compt. rend. Soc. de biöl., 1890, 42, p. 573.

²⁷ Ann. de l'Inst. Pasteur, 1895, 9, p. 620.

²⁸ La Cellule, 1845, 11, p. 177.

²⁹ Jour. Path. & Bacteriöl., 1896, 3, p. 39.

³⁰ Centralbl. f. allg. Path. u. path. Anat., 1898, 9, p. 827.

³¹ Compt. rend Soc. de biöl., 1890, 42, p. 646.

observations of Roemer,³² von Dungern,³³ Wassermann,³⁴ Hektoen,³⁵ and more recently, Besredka.³⁶ It would take us too far afield to discuss these studies at this point, but at all events it may be said that the actual existence of a purely local immunity has never been satisfactorily proved.

In previous articles we have discussed rather fully the form of experimental streptococcus empyema, which is produced regularly in rabbits by means of a strain of *Streptococcus pyogenes* ("H") originally obtained from a case of human empyema following measles and subsequently raised in virulence for rabbits by repeated passage through the pleural cavity of these animals. Although at first it produced a purulent and fatal empyema in rabbits only irregularly and in relatively large doses, the passage strain from the fourth generation on caused a characteristic and eventually fatal syndrome in every instance, even when very small amounts were used. One-tenth or 0.2 c c of a 24-hour broth culture of this organism, obtained immediately from the pleural fluid of a rabbit previously dead of the disease, is always fatal in from 4 to 5 days. Much smaller amounts are usually fatal, the minimal lethal dose apparently being 0.001 of a c c. The virulence of this passage strain is found not to fluctuate when we employ the pleural fluid from a fatal rabbit empyema as a standard source of culture. This pleural fluid contains living and virulent streptococci for several months when kept in the icebox, and has never in our experience become contaminated, in spite of repeated and often not overcareful opening of the tube for the inoculation of mediums. We do not, however, use any given pleural fluid for a period longer than one month. Although at first only 5% rabbit serum broth cultures were employed for inoculation, equally good results in the same doses have been obtained by using plain infusion broth of a p_H of from 7.4 to 7.8.

This empyema strain shows no distinct evidence of an elective affinity for the pleural cavity when administered intravenously. The passage pleural culture given by this route kills of a general septicemia with or without localized arthritis, but only in a minimal dose distinctly larger (0.01 to 0.1 c c) than by intrapleural inoculation. One-half of a cubic centimeter of bloody serous fluid was indeed found in the pleural cavity of one out of 19 animals that died after intravenous injection of various doses of pleural passage culture, and this fluid gave positive cultures of streptococcus. The exudate, however, showed little resem-

³² Arch. f. Oph., 1902, 54, p. 99.

³³ Die Antikörper, 1903.

³⁴ Ztschr. f. Hyg. u. Infektionskr., 1905, 50, p. 331.

³⁵ Jour. Infect. Dis., 1911, 9, p. 103.

³⁶ Ann. de l'Inst. Pasteur, 1919, 33, pp. 301 and 882; 1920, 34, p. 361; 1921, 35, p. 422.

blance to the extensive purulofibrinous exudate which regularly follows the injection into the pleura. Several intraperitoneal injections have been accompanied by pleurisy, which can be explained on the basis of simple extension, as in the usual involvement of the pericardium following the intrapleural doses.

When a small amount (0.1 c c) of the pleural passage culture of *S. pyogenes* "H" is injected intradermally near the midline on the back in any region from the scapula posteriad, a uniform erysipelas-like involvement of the regional lymphatics follows; this dose, however, is rarely fatal. With a larger dose (0.2 c c) the local reaction is also pronounced, and the animal dies of general septicemia. With smaller doses or with less virulent cultures, either only a slight reddening or a small pustule occurs. These observations correspond to the earlier investigations of the specificity of the erysipelas streptococcus by the observers to whom reference has been made. The original stock culture "H" from which our passage culture was derived and which has been conserved on rabbit blood agar for several years, produces no lesion intracutaneously with the regular doses. The results on which these statements are based are summarized in table 1.

TABLE 1

ERYSIPELAS PRODUCED BY PASSAGE "H" AND STOCK "H" CULTURES OF STREPTOCOCCUS FOLLOWING INTRADERMAL INJECTION IN VARIOUS PARTS OF THE BACK IN RABBITS

	Dose, C c	Total Number of Rabbits	Positive Lesion	Percentage	Death
Stock culture "H".....	0.2	9	1	11.1	
Stock culture "H".....	0.1	4	0	0	
Passage culture "H".....	0.2	8	8	100	7 (87.5%)
Passage culture "H".....	0.1	23	22	95.6	2 (9%)

In our experience inoculation on the back gives a much more definite and regular lesion than inoculation intracutaneously on the ear, where irregular and localized reactions were produced with 0.1 c c of our passage culture. A typical erysipelas involving the entire ear and lasting for several weeks follows the injection of 0.2 c c of the culture and is not followed by death, as when given on the back.

We are able then with regularity, or at least in over 95% of cases, to produce a characteristic erysipelatous lesion on the back of rabbits with 0.1 c c of the passage culture. Following the intracutaneous inoculation near the midline of the back, a red, hot, indurated area is evidenced at the end of 24 hours, which in 2 or 3 days extends to about

one-half the distance toward the midline of the abdomen. It takes the form of an indurated ribbon measuring from 2 to 3 cm. in width, with sharply raised margins, which spreads down the lymphatics, but not laterally, so that by the third to the fifth day it has reached the midline of the abdomen and then spreads anteriorly for from 2 to 5 cm. The lesion then remains stationary for about 2 weeks, during which period it usually breaks down in the center at one or more points and on pressure exudes a few drops of pus, which on culture is found to contain the streptococcus. The lesion then gradually contracts, is covered with brownish scales and eventually heals in from 3 to 4 weeks with no marked cicatrization and an almost complete return to normal condition. We have found in the few cases studied that the temperature rises from 1 to 3 degrees for the first 2 days following the intradermal injection. This fever, which is intermittent, is accompanied by a moderate increase of the polymorphonuclear leukocytes.

As already stated, when the larger dose of 0.2 c c of a 24-hour culture instead of 0.1 c c was employed, 7 of 8 animals so inoculated died in periods of from 24 hours to 20 days. In 2 animals only out of 23 has a fatal result followed the smaller dose. We have then in 0.1 c c of a 24-hour infusion broth passage culture a symptomatic, but rarely fatal, dose and in 0.2 c c a fatal dose. At this point a comparison of the minimal lethal dose by the different routes we have employed, intradermal, intravenous and intrapleural, is of interest as bearing again on the possible elective specificity of the passage pleural strain.

TABLE 2
MINIMAL LETHAL DOSE OF STREPTOCOCCUS PYOGENES "H," BOTH "STOCK" AND "PLEURAL
PASSAGE" CULTURES FOR RABBITS BY DIFFERENT ROUTES

Route	Stock "H" C c	Passage "H" C c
Intradermal.....	Negative to 0.2	0.2
Intravenous.....	Negative to 10	0.1
Intrapleural.....	0.1	0.001

Certain interesting conclusions appear from inspection of table 2:

In the first place, the strain of streptococcus "H," both stock and passage, is very much more pathogenic when administered by the pleura than by either the intradermal or the intravenous route; and second, it has been raised in virulence markedly by passage through the pleura, but so far as can be observed, there is no selective increase in pathogenicity, that is to say, the pleural passage strain is relatively no more

pathogenic by the pleural route than by either of the other 2 routes. The stock culture was not tested by the intradermal or intravenous route in doses larger than those that have been indicated, and it should be stated that the dose of 0.2 c c of stock culture intradermally not only is not fatal, but does not produce the characteristic erysipelas.

It is of interest here to recall the variations that have so far been observed between the original "stock" and "passage" cultures of the strain of the streptococcus with which we have been working in this laboratory for the past several years. The passage culture differs from the stock in the following particulars:

1. It is more pathogenic for rabbits, irrespective of route, as outlined in the foregoing.

2. It has an increased ability to produce hemotoxin (Cook, Mix and Culvyhouse ⁴).

3. It shows a marked difference in nitrogen metabolism in its early growth in certain mediums. This is indicated in the passage strain by a decrease in amino acid output, coupled with increased ammonia excretion, while the stock strain shows the opposite set of conditions (Foster ⁷).

IMMUNITY FROM EXPERIMENTAL ERYSIPELAS

Rabbits that have recovered from the intradermal symptomatic dose of the passage culture, that is to say, from experimental erysipelas, have been tested at different intervals for their protection against subsequent inoculation, and the results are briefly recorded in table 3.

TABLE 3
PROTECTION AGAINST ERYSIPELAS AFFORDED BY ONE OR MORE INJECTIONS OF A SYMPTOMATIC DOSE OF PASSAGE CULTURE INTRADERMALLY

Number of Animals	Days After First Inoculation Tested	Protected	
		Number	Percentage
3.....	7- 14	0	0
19.....	20-141	17	89.4

It appears that a definite immunity is produced by recovery from experimental erysipelas as tested 3 or more weeks following the primary inoculation. The 2 unprotected animals were tested the second time 60 and 141 days after the primary inoculation, which suggests that the immunity acquired is temporary, although, as the table shows, one animal at least was protected for 3½ months. A word should be said as

to what constitutes our criterion of protection against this experimental lesion as we have described it. It was found after a number of measurements that the average distance from the vertebrae to the midabdominal line in rabbits such as we have employed in these experiments, that is to say, from 2,000 to 3,500 gm. in weight, is on an average 16 cm. An extension of the lesion to this point has been designated as a triple plus or complete reaction (+++); extension for one-half the distance, i. e., about 8 cm., a double plus reaction (++) , and anything less than this distance is designated as a single plus reaction (+). In all but one of the 23 control animals, the inoculation extended to a ++ degree, and in most instances the entire distance (+++), actually in 15 animals of 23. We have, therefore, adopted a double plus reaction, that is, extension one-half way to the midline, as evidence of a failure of protection, and the figures indicated in table 3 are based on this criterion. A temporary local induration lasting for 2 or 3 days and approaching a + reaction may be obtained with stock "H" culture or other nonpathogenic cultures of streptococcus or even with streptococcus vaccines.

The animals that were tested for protection have in all instances received the second test inoculation at a different point from the one utilized in the first inoculation, that is to say, when the first positive lesion was obtained on the right side of the body, the second inoculation was given to the left of the vertebral column. It would not seem, then, as might be indicated from the work of earlier investigators, particularly Cobbett and Melsome,^{29,30} that protection is present only in a locally immunized or simply an inflamed area. Furthermore, inoculation over the scapular region protects perfectly well against second inoculation several inches posteriad on the other side. Primary inoculations of 0.1 c c in the ear which do not produce a well-defined lesion, as we have already mentioned, do not protect, but a dose of 0.2 c c in that locality produces a marked lesion without death and gives perfect protection against a second inoculation on the back. The degree of primary reaction then is of importance in determining protection. This is concretely evidenced from our results on reinoculation of a symptomatic dose in a number of animals which on primary intradermal injection of this dose into the back had shown no characteristic lesion, owing to previous treatment by serum or vaccine. Six out of 8 such animals, when tested between the 26th and 76th day following the initial dose of passage culture, showed no protection against erysipelas. The fact that such animals had not reacted to the first symptomatic dose of course shows

some degree of protection from the vaccines themselves and logically introduces the question as to whether animals can be protected in any other way than by recovery from erysipelas.

The next point to consider is the protection that may be afforded by stock cultures which produce small lesions or none at all; by subsymptomatic doses of the passage culture; and by vaccines prepared from the passage culture. In tables 4 to 7 are summarized the results of these experiments.

The data embodied in these tables will be understood with the following brief statement:

(1) Living Cultures of Stock Streptococcus "H": These may be inoculated repeatedly in doses at least as large as 0.2 c c of a 24-hour injection broth culture intradermally without producing more than a slight local induration. The number of injections and the amounts employed are indicated in the table.

(2) Saline Passage "H" Vaccines: Infusion broth was inoculated with passage streptococcus "H," incubated for 48 hours, centrifugalized and the sediment suspended in absolute alcohol, shaken, recentrifugalized, suspended in ether and dried over CaCl_2 to constant weight. This dried culture was then ground in an agate mortar² and suspended in 0.5% phenolated saline solution in such volume that 1 c c contained 10 c c of the original culture fluid.

(3) Oil Passage "H" Vaccines: Killed and dried by alcohol and ether parallel to the saline vaccines, but suspended in 0.5% phenolated cotton seed or olive oil.

The following conclusions may be drawn from the experimental evidence presented in tables 4 to 7.

It is evident that 0.1 c c of the passage culture is about the smallest amount that will produce a characteristic erysipelas, as we have defined and limited it. At least, 0.01 c c and less fails to do so. A barely subsymptomatic dose of living culture, 0.01 c c, may produce protection (rabbit 553), but a smaller dose 0.001 c c, does not.

It is clear from rabbit 550 and table 6 that ordinary heat killed or alcohol killed vaccine, whether sensitized or plain, does not protect even when given repeatedly. Oil vaccines prepared in the same general way (table 7) do, however, protect in the majority of cases.

Living stock cultures of "H," which in itself produces no lesion, will, when given repeatedly, protect against the passage culture. Apparently one or two injections do not usually suffice to protect, as when a lesion is produced with a passage culture.

TABLE 4

RESULTS IN FOUR RABBITS GIVEN DIFFERENT PREPARATIONS OF PASSAGE STREPTOCOCCUS "H" AND FOLLOWING AN INTERVAL OF 28 DAYS TESTED WITH A SYMPTOMATIC DOSE OF PASSAGE CULTURE STREPTOCOCCUS "H," 0.1 C C, FOR PROTECTION AGAINST ERYSIPELAS

Number	First Inoculation	First Lesion	Protection on Second Inoculation
558	0.1 c c Passage "H".....	++	Nearly complete
553	0.01 c c Passage "H".....	±	Complete
551	0.001 c c Passage "H".....	+	None
550	0.1 c c Passage "H" 65 C.....	0	None

TABLE 5

RESULTS IN RABBITS GIVEN VARIOUS AMOUNTS AND NUMBER OF DOSES OF LIVING STOCK CULTURE "H" AND TESTED SUBSEQUENTLY WITH THE SYMPTOMATIC DOSE OF PASSAGE CULTURE

Rabbit	Route	Number Injections	Billions	Duration	Lesions	Protection
760	Intradermal	5 living stock "H"	0.5	55	0	+
823	Intradermal	4 living stock "H"	0.004	30	0	+
646	Intradermal	3 living stock "H"	0.6	21	+	+
765	Intradermal	3 living stock "H"	0.3	39	0	+
562	Intradermal	2 living stock "H"	0.2	31	+	+
742	Intradermal	2 living stock "H"	0.2	31	+++	0
552	Intradermal	1 living stock "H"	0.1	28	+++	0
573	Intradermal	1 living stock "H"	0.2	28	++	Receding ±*
574	Intradermal	1 living stock "H"	0.2	28	+++	0
575	Intradermal	1 living stock "H"	0.2	28	+++	0
576	Intradermal	1 living stock "H"	0.2	28	0	+

* In 2 instances in incompletely immunized animals, a lesion progressed to ++ in 3 or 4 days and then receded. This never occurs in the normal animal, and we regard it as evidence of partial protection.

TABLE 6

RESULTS IN RABBITS GIVEN VARIOUS AMOUNTS AND VARYING NUMBER OF INJECTIONS OF KILLED VACCINES, SENSITIZED AND UNSENSITIZED, AND SUBSEQUENTLY TESTED FOR PROTECTION AGAINST ERYSIPELAS

Rabbit	Route	Number Injections	Billions	Duration	Lesions	Protection
626	Intradermal	4 sensitized vaccine	2	22	+++	0
625	Intradermal	4 sensitized vaccine	2	22	+++	0
891	Intradermal	5 plain vaccine	2.5	26	+++	0
657	Intradermal	3 plain vaccine	1.5	29	+++	0
660	Intradermal	3 plain vaccine	1.5	29	+++	0

TABLE 7

RESULTS IN RABBITS GIVEN VARIOUS AMOUNTS AND A VARYING NUMBER OF INJECTIONS OF KILLED OIL VACCINES AND TESTED SUBSEQUENTLY FOR PROTECTION AGAINST ERYSIPELAS

Rabbit	Route	Number Injections	Billions	Duration	Lesions	Protection
656	Intradermal	3 oil vaccine	1.5	29	0	+
524	Intradermal	3 oil vaccine	3	28	+	+
688	Intradermal	3 oil vaccine	3	28	+++	0
628	Intradermal	3 oil vaccine	1.5	21	0	+
663	Intradermal	2 oil vaccine	1	22	+++	0

The failure of ordinary vaccine to protect against erysipelas is consonant with other results that we have obtained in immunizing against intravenous or intrapleural infection with the streptococcus; namely, that living cultures produce a much more marked grade of active immunity than do killed cultures. It is evident in the immunization against erysipelas that oil vaccine is less rapidly absorbed from local areas than a saline suspension. This fact, among others, suggests that this particular immunity is to some extent at least a local phenomenon and suggests other methods of comparison to settle these points. It has appeared to us for some time in the course of these investigations on the different routes of immunization and infection with the streptococcus that local phenomena are important; for instance, we have apparently been able to immunize against pleural infection when vaccines followed by small amounts of living stock cultures have been given into the pleura, but not when the same amounts were injected intravenously or intradermally. We are not as yet able to present wholly convincing evidence of the superiority of local intrapleural immunization, owing to a failure in the majority of our early experiments to obviate the marked differences in the infecting dose by the different routes. The test doses of our passage culture are practically the same by intravenous and intradermal routes, that is to say, 0.1 c c intradermally gives erysipelas without death, and intravenously is fatal.

In table 8 are displayed the histories of 8 rabbits that were immunized by intravenous injections of killed streptococcus vaccines, followed by living stock or passage cultures of streptococcus "H." They are all animals which would usually have been found to be unaffected by a test dose (0.1 or 0.2 c c) of passage culture administered intravenously. They would moreover all, except perhaps animal 120, have been protected against erysipelas if the immunizing injections had been given intradermally, and yet when tested by intradermal injections of 0.1 c c of passage "H," 5 of the 8 (62.5%) developed characteristic lesions.

In table 9 are listed 11 animals which were immunized by the intradermal route and then actually proved to be protected against erysipelas. They were then injected intravenously with the same dose of the same passage culture and only 7 of the animals survived. Here the chances were against demonstration of a difference in protection between intradermal and intravenous routes, because the test intradermal dose of course increased the immunization. Four other animals that were not given the intradermal test, but that by comparison were protected against erysipelas, all died on intravenous injection.

Rabbits that have recovered from several carefully graded intrapleural injections, first of vaccine and then small amounts of living stock culture, are found to be protected not simply against multiple fatal doses intrapleurally, but against intradermal erysipelas as well.

All these experiments we advance simply as indicative that there really exists a local immunity as contrasted with a general immunity. Complete demonstration of this fact must await extensive comparative series of animals immunized by the various routes and subsequently

TABLE 8

PROTECTION AGAINST EXPERIMENTAL ERYSIPELAS AFFORDED BY INTRAVENOUS IMMUNIZATION; 0.1 C C OF PASSAGE "H" CULTURE GIVEN INTRADERMALLY AS A TEST

Rabbit	Number and Kind of Injections	Dose in Billions	Duration, Days	Lesions	Protection
120	14 dead saline vaccine.....	14	100	+++	0
506	3 oil vaccine.....	7.5	38	0	+
611	2 killed sensitized vaccine.....	7.5			
	3 living stock "H".....	+0.0003 c c	41	+++	0
612	Idem.....	Idem	41	+++	Death 0
697	3 oil vaccine.....	7.5			
	1 living passage "H".....	+0.2	60	0	+
818	4 living stock "H".....	0.004	20	++	0
832	1 living passage "H".....	0.01	97	0	+
827	1 living passage "H".....	0.2	37	+++	0

TABLE 9

PROTECTION OF RABBITS AGAINST A FINAL INTRAVENOUS INJECTION OF 0.1 C c OF PASSAGE "H" *

Rabbit	Number of Injections	Dose in Billions	Duration	Result
763	6	0.6	12 weeks	Recovered
622	1	0.1	3 weeks	Recovered
663	4	2.2	8 weeks	Recovered
837	5	0.5	19 weeks	Dead, 14 days
823	5	0.5	7 weeks	Recovered
822	4	0.4	4 weeks	Dead, 3 days
641	9	0.9	10 weeks	Recovered
742	8	0.8	15 weeks	Recovered
660	5	0.2	8 weeks	Recovered
646	5	0.9	11 weeks	Dead, 6 days
833	5	0.5	19 weeks	Dead, 11 days

* Living stock cultures were used to immunize and perfect protection against erysipelas was assured by intradermal injection of 0.1 c c of passage culture before the intravenous test was given.

tested simultaneously by each of these routes. The difficulties of this demonstration are not simply in the number of animals required, but in carefully equilibrating the multiple of lethal doses employed in accordance with the route utilized. Furthermore, to demonstrate local immunity, precisely the right degree of protection must be attained and no more, or the local immunity will pass over into a general form.

CONCLUSIONS

The passage pleural culture of *Streptococcus pyogenes* ("H"), with which we have been working for several years, has acquired new and relatively constant properties. In addition to certain changes in its metabolic functions, it has gained a marked pathogenicity for rabbits, whereas the original stock culture has little. Constant empyema is produced by direct inoculation of very small amounts of the passage strain. In considerably large doses (about 100 times) it will usually produce a fatal septicemia when given intravenously and a recoverable erysipelas when injected intradermally in the back. Somewhat larger doses are necessary to produce erysipelas in the ear. This constant lesion is not produced by the stock culture.

Recovery from erysipelas from a period roughly of 3 weeks after inoculation confers complete protection against intradermal reinoculation, irrespective of its locality. This protection usually lasts for at least 3 months. Repeated injections of heat or alcohol killed vaccines from the passage strain do not protect against the local lesion. Oil vaccines in the same amount frequently protect. Several injections of the original living stock culture, which produces no lesion, protect against the passage strain.

The fatal intravenous dose and the symptomatic intradermal dose of the passage culture are practically the same. Complete protection against intradermal inoculation by previous intradermal infection or immunization frequently does not protect against intravenous infection. The reverse set of conditions apparently also prevails; that is, intravenous immunization protects better against intravenous than against intradermal infection.

These last facts, together with other indications from our own experiments and from observations of others, point strongly to the existence of a true local tissue immunity following local streptococcus infection or immunization.

THE ADAPTATION OF THE HEIST-LACY METHOD FOR DETERMINING THE BACTERICIDAL ACTIVITY OF WHOLE BLOOD FOR CHEMOTHERAPEUTIC INVESTIGATIONS

STUDIES IN THE CHEMOTHERAPY OF BACTERIAL INFECTIONS. I

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In chemotherapeutic investigations in bacterial infections, test tube bactericidal tests are of service for detecting evidences of specific bacteriotropism and for furnishing "leads" in the preparation of new compounds; when these tests are conducted with serum or blood they may indicate the important influence of these substances on bactericidal activity, but they are no index to the degree of bactericidal activity occurring in vivo when administered intravenously to infected living animals and do not measure the degree of organotropism or toxicity for body cells.

Tests conducted in vivo with experimental infections may yield negative results because the drug may be of such toxicity or high organotropism that an amount sufficient to exert a demonstrable degree of bactericidal activity or parasitropism cannot be administered; notable examples are various mercurial compounds possessing marked bactericidal activities in the test tube even in serum and blood, but proving too toxic for the living animal to permit the administration of an amount sufficient to influence materially a generalized infection or bacteremia. Even if a compound may be administered in an amount sufficient to increase the bactericidal activity of the blood for a certain bacterium, the micro-organisms may proliferate so rapidly or gain access to the blood in such numbers that the feeble but positive bactericidal activity of the compound may be masked and undetermined. This is apparently true of ethylhydrocuprein (optochin) hydrochlorid in the treatment of lobar pneumonia, as shown by the studies of Moore and Chesney.¹

Experimentally produced local infections of serous cavities provide more sensitive lesions for chemotherapeutic studies in vivo than bacteremias or generalized infections. Kolmer and Idsumi² found that

Received for publication, April 24, 1922.

¹ Arch. Int. Med., 1917, 19, p. 611; 1918, 21, p. 659.

² Jour. Infect. Dis., 1920, 26, p. 355.

subtoxic amounts of ethylhydrocuprein (optochin) hydrochlorid injected into the subarachnoid space of the spinal cord may favorably influence the course of pneumococcus meningitis in rabbits; in a similar study with Sands³ in experimental pneumococcus pleuritis of guinea-pigs, the results were more decisive. These investigations have indicated that subtoxic amounts of ethylhydrocuprein hydrochlorid injected into these cavities may be sufficient to exert marked pneumococcidal activity even when the drug is diluted with the inflammatory exudates. The degree of dilution, however, is probably never as great as when the drug is introduced into the blood stream and hence exerts more marked curative activity in an infected cavity before being absorbed than is possible in the treatment of a bacteremia or generalized infection.

In vivo experiments are usually prolonged, laborious and expensive and require frequent repetition before reliable results are obtained. Of greater importance, however, is the fact that minor degrees of specific parasitropism of new compounds may not be elicited for the reasons previously mentioned, and thereby fail to furnish data for the guidance of chemotherapeutic studies. For this reason a combined in vivo-in vitro test may prove of value when conducted by administering the drug in sublethal or subtoxic amounts to animals, securing small amounts of blood at intervals and measuring the bactericidal activity of the whole blood or serum in vitro. By means of plating methods Moore and Chesney¹ were able to show that ethylhydrocuprein (optochin) hydrochlorid administered intravenously, intramuscularly or orally may raise the pneumococcidal activity of the serum even though the drug is too toxic to permit the administration of an amount sufficient to raise this bactericidal activity to the range of therapeutic activity in pneumococcus pneumonia.

In a search for a reliable technic for measuring the bactericidal activity of the whole blood adaptable for use on a large scale in the course of chemotherapeutic investigations with the lower animals, we have given the Heist-Lacy method an extensive trial, and the purpose of this paper is to summarize the results observed.

The technic has been described by Heist, S. Solis-Cohen and M. Solis-Cohen.⁴ The essential feature of the method, suggested by Benjamin F. Lacy, is that a capillary glass tube is filled, by capillary attraction, up to a fixed mark, with a broth culture of the bacterium, and then emptied. A certain number of bacteria remain sticking to

³ Jour. Exper. Med., 1921, 33, p. 693.

⁴ Jour. Immunology, 1918, 3, p. 261.

the wall of the tube. Blood as it comes from capillary or vein is allowed to flow up the tube to the mark, and the tube is then sealed and incubated. If the blood has no bactericidal action the bacteria which have remained on the wall of the tube find themselves in a favorable medium and multiply rapidly: If it is bactericidal, they are killed and no growth results. Readings are made by blowing out the contents of the tube on a glass slide and staining and examining them under the microscope. By combining several capillary tubes into one many-stemmed pipet, modeled after the one Wright uses for estimating the coagulation time of the blood, and by using a series of ascending dilutions of broth culture, an approximate quantitative value may be given to the test.

By means of this method, which is remarkably simple as compared with the usual plating bactericidal tests and employs whole blood instead of serum, Heist, Cohen and Cohen⁴ were able to demonstrate the high pneumococcal activity of the blood of the pigeon in contradistinction to the feeble or absent pneumococcal activity of the blood of the rabbit. They were also able to measure the degree of active immunity resulting from immunization of rabbits with pneumococci of different types.⁵ Matsunanni and Kolmer found that the test elicited varying degrees of natural immunity to virulent meningococci,⁶ corroborating the observations of Heist, Cohen and Cohen, that the results of this *in vitro* test tend to run parallel with the degree of immunity elicited by injecting animals with virulent cultures of bacteria.

EXPERIMENTAL

Our experiments were conducted by determining the bactericidal activity of the blood of normal rabbits before and at varying intervals after the administration of several compounds.

Two micro-organisms were employed: a highly virulent type 1 pneumococcus and a culture of *Staphylococcus aureus*. Heist has emphasized the importance of using young broth cultures of virulent pneumococci; our experiments were conducted with 18-hour cultures in the hormone broth described by Huntoon. As shown in the tables, 5 dilutions made with hormone broth were employed: 1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000.

As stated, the test is conducted with the micro-organisms adhering to the interiors of the capillary tubes; the actual numbers vary according to the density of the parent growth but with a uniform broth remains fairly constant. In order to minimize this error and render different experiments more comparative, Heist has adopted the practice of making large steps in preparing the dilutions of culture.

⁴ Heist, G. D., and Cohen, S. Solis: *Ibid.*, 1919, 4, p. 147.

⁶ *Ibid.*, 1918, 3, p. 201.

By washing out the tubules with broth and plating these fluids Heist has found that the actual numbers of viable staphylococci adhering to the interiors of the capillary tube were approximately as follows:

Staphylococcus	
Undiluted.....	1900
1: 10.....	500
1: 100.....	100
1: 1,000.....	20
1: 10,000.....	8

The majority of the experiments were conducted with ethylhydrocuprein (optochin) hydrochlorid, other quinine compounds such as the bisulphate, hydrochlorid and quinine and urea hydrochlorid; mercurophen (sodium oxy-mercury-ortho-nitro phenolate) and mercuric chlorid as a control on the results observed with mercurophen.

Healthy adult rabbits weighing from 1,800 to 2,400 gm. were employed. Blood was secured from ear veins after cleansing and contaminations did not occur confirming the observations of Heist, Cohen and Cohen⁴ that non-pathogenic bacteria do not grow in whole blood unless comparatively large numbers are seeded.

Preliminary tests were always made, but the whole blood of rabbits was found practically without bactericidal activity for the cultures of pneumococci and staphylococci employed in this study.

The medicaments were administered intravenously, subcutaneously and by stomach tube in doses according to body weight. The bactericidal tests were then repeated at varying intervals. In some experiments the medicaments were given once daily for 4 days, the bactericidal tests being conducted 24 hours after each administration and just before the succeeding dose. After the fourth dose daily bactericidal blood tests were conducted until the influence of the medicament had disappeared. In every experiment culture controls with broth were set up each day and always yielded heavy growths.

RESULTS OBSERVED WITH OPTOCHIN (ETHYLHYDROCUPREIN) HYDROCHLORID

(a) *Single Intravenous Injection*.—As shown in tables 1 and 2, the intravenous injection of 10 and 20 mg. of ethylhydrocuprein hydrochlorid per kilo of body weight imparted a decided pneumococcal activity to the blood of rabbits. These amounts were approximately $\frac{1}{6}$ to $\frac{1}{4}$ the highest tolerated doses by slow intravenous injection.

This increased bactericidal activity was apparent within a few minutes after each injection, but appeared to increase slightly during the following 24 hours, to be followed by a sharp decline.

Duplicate tests made at the same time with a culture of *Staphylococcus aureus* showed a slight increase in bactericidal activity particularly with the 20 mg. dose of the drug. These effects were apparent, however, only with the higher dilutions of the culture, the greater bactericidal activity of the drug for the pneumococcus standing out in sharp contrast.

(b) *Multiple Intravenous, Subcutaneous and Oral Administrations*.—The pneumococcal activity of the blood of a series of rabbits was tested on each of 2 succeeding days and found practically absent even with 1:100,000 dilutions of culture. Ethylhydrocuprein hydrochlorid was then administered intravenously in a dose of 10 mg. per kilo; subcutaneously in a dose of 20 and 30 mg. per kilo and by stomach tube in a dose of 25 and 40 mg. per kilo. The drug was given once a day for 4 days and the blood tests repeated daily. The results are summarized in table 2.

Twenty-four hours after the first dose the whole blood had acquired well defined pneumococidal properties even after the oral administration of the drug, which confirms the observations of Moore and Chesney.¹ With the succeeding doses the bactericidal effects became more marked. After the last dose (fourth) these bactericidal activities promptly diminished and were usually absent two or three days later.

TABLE 1

THE INFLUENCE OF ONE INTRAVENOUS INJECTION OF ETHYLHYDROCUPREIN HYDROCHLORID IN A DOSE OF 0.01 GM. PER KILO ON THE BACTERICIDAL ACTIVITY OF THE BLOOD OF RABBITS FOR PNEUMOCOCCI AND STAPHYLOCOCCI

Blood Tests	Pneumococcus Type 1					Staphylococcus aureus				
	1:10*	1:100	1:1000	1:10,000	1:100,000	Undil.*	1:10	1:100	1:1000	1:10,000
Before injection.....	++	++	++	++	++	++	++	++	++	++
Immediately after.....	++	++	—	—	—	++	++	++	++	++
1 hour after.....	++	—	—	—	—	++	++	++	++	++
4 hours after.....	++	+	—	—	—	++	++	++	++	++
24 hours after.....	++	—	—	—	—	++	++	++	—	—
48 hours after.....	++	++	++	++	++	++	++	++	++	++
Culture controls.....	++	++	++	++	++	++	++	++	++	++

* Dilutions of 18-hour broth cultures.

+ = heavy growth; ++ = slight growth; — = no growth.

TABLE 2

THE INFLUENCE OF MULTIPLE ADMINISTRATIONS OF ETHYLHYDROCUPREIN HYDROCHLORID ON THE PNEUMOCOCCIDAL ACTIVITY OF THE BLOOD OF RABBITS

Blood Tests	Intravenously; 10 Mg. per Kilo					Subcutaneously; 20 Mg. per Kilo					By Mouth; 40 Mg. per Kilo				
	1: 10*	1: 100	1: 1,000	1: 10,000	1: 100,000	1: 10	1: 100	1: 1,000	1: 10,000	1: 100,000	1: 10	1: 100	1: 1,000	1: 10,000	1: 100,000
First preliminary.....	++	++	++	++	+	++	++	++	++	++	++	++	++	++	++
Second preliminary.....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
24 hours after first dose.....	+	—	±	—	—	+	—	—	—	—	+	+	—	—	—
24 hours after second dose.....	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
24 hours after third dose.....	—	—	—	—	—	—	—	—	—	—	++	++	—	—	—
24 hours after fourth dose.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
48 hours after fourth dose.....	++	++	++	++	++	++	++	+	+	+	++	++	+	—	—
72 hours after fourth dose.....	++	++	++	+	—	++	++	++	+	±	++	+	+	—	—
96 hours after fourth dose.....	++	++	++	++	++	++	++	++	++	+	++	++	++	++	++
Culture controls.....	++	++	++	++	++	++	++	++	++	+	++	++	++	++	++

* Dilutions of 18-hour broth cultures.

RESULTS OBSERVED WITH OTHER CINCHONA COMPOUNDS

As shown by Cohen, Kolmer and Heist⁷ and others, many of the ordinary compounds of quinine are pneumococidal, particularly in vitro. None, however, have been found equal to ethylhydrocuprein hydrochlorid either in vitro or in protective and curative tests with infected mice.

⁷ Jour. Infect. Dis., 1917, 20, p. 272.

In order to determine the value of the Heist-Lacy method for eliciting varying degrees of bactericidal activity, three quinine compounds have been tested and the results compared with those observed with ethylhydrocuprein hydrochlorid.

The compounds were administered intravenously in a dose of 10 mg. per kilo; subcutaneously in a dose of 30 mg. per kilo and by stomach tube in a dose of 25 mg. per kilo. (The results are illustrated in table 3.)

All of the 3 compounds employed, namely, quinine and urea hydrochlorid, quinine bisulphate and quinine hydrochlorid, imparted a definite pneumococcal activity to the blood by all 3 routes of administration. These effects were slightly better after intravenous injection than after subcutaneous and oral administration. The effects produced by the 3 drugs were closely similar although the hydrochloride, which contains about 81% of the alkaloid, was

TABLE 3

THE INFLUENCE OF MULTIPLE ADMINISTRATIONS OF QUININE HYDROCHLORIDE ON THE PNEUMOCOCCAL ACTIVITY OF THE BLOOD OF RABBITS

Blood Tests	Intravenously; 10 Mg. per Kilo					Subcutaneously; 30 Mg. per Kilo					By Mouth; 25 Mg. per Kilo				
	1: 10*	1: 100	1: 1,000	1: 10,000	1: 100,000	1: 10	1: 100	1: 1,000	1: 10,000	1: 100,000	1: 10	1: 100	1: 1,000	1: 10,000	1: 100,000
First preliminary.	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+
Second preliminary.	++	++	++	+	+	++	++	++	++	++	++	++	++	++	++
24 hours after first dose.	++	++	++	+	+	++	++	++	++	0	++	++	++	++	++
24 hours after second dose.	+	—	—	—	—	+	+	+	—	—	—	—	—	—	—
24 hours after third dose.	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—
24 hours after fourth dose.	++	++	++	++	+	++	++	+	+	—	++	+	+	—	—
48 hours after fourth dose.	++	++	++	++	++	++	++	++	++	++	++	++	++	+	—
72 hours after fourth dose.	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Culture controls.	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

* Dilutions of 18-hour broth cultures of pneumococcus type 1.

slightly superior to the bisulphate and quinine and urea hydrochlorid which contain about 58% alkaloid. All 3 were inferior to ethylhydrocuprein hydrochlorid which contains about 90% of alkaloid.

It was interesting to find, however, that the Heist-Lacy method is capable of yielding approximately quantitative results and that the administration of the common quinine compounds appreciably increases the pneumococcal activity of the blood, even when administered by mouth.

RESULTS WITH MERCUROPHEN AND MERCURIC CHLORID

Mercuriofen, which is a compound of mercury introduced by Schamberg, Kolmer and Raiziss,⁸ possesses an extremely high and selective bactericidal activity in vitro for cocci, especially for staphylococci. For members of the typhoid-colon group of bacilli its bactericidal activity is about the same or slightly inferior to mercuric chlorid.

As reported by Schamberg, Kolmer and Raiziss,⁵ the intravenous injection of mercurophen into rabbits in sublethal amounts (0.0015 gm. per kilo) was found to increase appreciably the bactericidal activity of the blood for a few hours for staphylococci, pneumococci and *B. typhosus*, whereas the administration of mercuric chlorid and other mercurial compounds usually failed to

TABLE 4

THE INFLUENCE OF ONE INTRAVENOUS INJECTION OF MERCUROPHEN IN A DOSE OF 0.0008 GM. PER KILO ON THE BACTERICIDAL ACTIVITY OF THE BLOOD OF RABBITS FOR PNEUMOCOCCI AND STAPHYLOCOCCI

Blood Tests	Pneumococcus Type 1					Staphylococcus aureus				
	1:10*	1:100	1:1000	1:10,000	1:100,000	1:10*	1:100	1:1000	1:10,000	1:100,000
Before injection.....	++	++	++	++	++	++	++	++	+	+
Immediately after.....	++	++	++	+	+	++	+	—	—	—
1 hour after.....	++	++	++	++	+	++	++	+	—	—
4 hours after.....	++	++	++	++	+	+	+	—	—	—
24 hours after.....	++	++	++	++	+	++	++	+	+	+
48 hours after.....	++	++	++	++	+	++	++	++	++	++
Culture controls.....	++	++	++	++	++	++	++	++	++	+

* Dilutions of 18-hour broth cultures.

TABLE 5

THE INFLUENCE OF ONE INTRAVENOUS INJECTION OF MERCUROPHEN IN A DOSE OF 0.0008 FOR PNEUMOCOCCI AND STAPHYLOCOCCI

Blood Tests	Intravenously; 5 Mg. per Kilo*					Subcutaneously; 10 Mg. per Kilo					Orally; 20 Mg. per Kilo				
	1: 10†	1: 100	1: 1,000	1: 10,000	1: 100,000	1: 10	1: 100	1: 1,000	1: 10,000	1: 100,000	1: 10	1: 100	1: 1,000	1: 10,000	1: 100,000
First preliminary.	++	++	++	++	+	++	++	++	++	++	++	++	++	++	++
Second preliminary.	++	++	++	++	+	++	++	++	++	++	++	++	++	++	++
24 hours after	++	++	++	++	+	++	++	++	++	++	++	++	++	++	++
first dose.....	++	++	+	—	—	++	++	++	—	—	+	—	—	—	—
24 hours after	++	+	+	—	—	+	+	—	—	—	+	—	—	—	—
second dose.....	++	+	+	—	—	+	+	—	—	—	+	—	—	—	—
24 hours after	+	—	—	—	—	+	+	+	—	—	+	—	—	—	—
third dose.....	++	+	—	—	—	++	++	+	—	—	++	+	—	—	—
48 hours after	++	++	++	++	++	++	++	++	++	++	++	+	+	+	+
fourth dose.....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
72 hours after	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Culture controls..	++	++	++	++	++	++	++	++	+	—	++	++	++	++	++
fourth dose.....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

* Rabbit died 96 hours after fourth dose.

† Dilutions of 18-hour broth cultures of pneumococcus type 1.

increase to the same degree the bactericidal activity of the whole blood of rabbits for these micro-organisms.

Similar results were observed in this study. The intravenous injection of 0.0008 gm. mercurophen per kilo, which is about $\frac{1}{8}$ the highest tolerated dose, increased the bactericidal activity of the blood for staphylococci but not for

⁵ *Ibid.*, 1919, 24, p. 547.

pneumococci (table 4). A similar dose of mercuric chlorid, which corresponded to about $\frac{1}{2}$ the highest tolerated dose, slightly raised the bactericidal activity of the blood for pneumococci.

As stated, *in vitro* tests have shown that mercurophen has a specially high bacteriostatic and bactericidal effect on staphylococci, and this superiority over mercuric chlorid was apparent in the results of the Heist-Lacy tests.

Mercurophen, however, also possesses pneumococcal activity when administered to rabbits intravenously, subcutaneously and by mouth. But large amounts are required to produce these effects.

Table 5 shows the results observed when multiple doses were given intravenously, subcutaneously and by mouth.

The amounts of mercurophen administered were approximately one-half the highest tolerated dose (not the MLD). As shown in this table, appreciable effects were apparent after the first dose, which increased after subsequent doses but rapidly disappeared when the administration ceased. The amounts of mercuric chlorid administered were practically the highest tolerated doses; a slight increase of the pneumococcal activity of the blood was apparent with the higher dilutions of culture, but the results were more irregular and less marked than observed with mercurophen.

SUMMARY AND CONCLUSIONS

In chemotherapeutic investigations in bacterial infections four procedures may be employed for evaluating a compound: (1) bacteriostatic and bactericidal tests *in vitro*; (2) a combined *in vivo-in vitro* test consisting of the administration of the compound in subtoxic amounts to animals and testing the bactericidal activity of the whole blood or serum at intervals; (3) the treatment of experimentally produced localized infections, such as meningitis and pleuritis, and (4) the treatment of experimentally produced generalized infections.

The Heist-Lacy method is a simple method for determining the bactericidal activity of whole blood at intervals after the administration of a drug and yields approximately quantitative results. If rabbits are employed, the test micro-organism should be one capable of surviving and proliferating in the blood of these animals, in order to elicit more clearly the possible bactericidal activity imparted by the drug under study.

The administration of ethylhydrocuprein (optochin) hydrochlorid to rabbits by intravenous, subcutaneous and oral routes results in rendering the whole blood decidedly pneumococcal. Similar but much less marked results followed the administration of other quinine compounds as the bisulphate, hydrochlorid and quinine and urea hydrochlorid. The bactericidal properties of the blood rapidly disappeared after the administration of these compounds had ceased.

By means of the Heist-Lacy method it was found that the administration of large amounts of mercurophen appreciably raised the bactericidal activity of the blood of rabbits for staphylococci and pneumococci, especially the former. The administration of mercuric chlorid was followed by slight and irregular increase of the bactericidal activity of the blood of living rabbits for these micro-organisms.

The Heist-Lacy method has been found less susceptible to contamination and less laborious than plating methods for measuring the bactericidal activity of whole blood. Since only a few drops of blood are required it permits the use of small animals and of repeated tests.

CULTURAL METHODS FOR THE GONOCOCCUS *

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In connection with a serologic study ¹ of the gonococcus it was necessary for us to devise methods and mediums whereby this organism might be isolated readily from gonorrheal infections, and also a large collection of strains might be maintained with a minimal risk of any of them being lost. The difficulties inherent in the cultivation of the gonococcus are evidenced by the great variety of mediums and procedures which have been recommended for this purpose, especially within recent years. At present there seems to be no consensus of opinion as regards the best methods to use. In the following section certain simple mediums are described which have given good results consistently in connection with the various purposes specified. It may be remarked that through the use of a sterilizable medium of proper reaction hardly greater difficulty has been encountered in maintaining a large collection of gonococcus strains than would be associated with carrying a like number of typhoid strains.

In another article ² are reported the results obtained through the application of these methods to the isolation of gonococcus strains from cases of mild chronic gonorrhea in women, together with a comparative study of the relative value of the cultural, complement-fixation and smear methods of diagnosis of such cases.

MEDIUMS

As a matter of convenience, the mode of preparation of certain of the mediums employed in these experiments will be described here, leaving the discussion of their uses and particular advantages to subsequent sections.

A. Ascitic-veal-urine-glycerol-agar. This plating medium, especially when combined with the dye, iodine-green, was found effective in the isolation of the gonococcus. The Thalmann ³ method of preparation has been followed to some extent, but the formula has been materially modified.

Received for publication, April 24, 1922.

* This study was aided by a grant from the U. S. Interdepartmental Social Hygiene Board, Washington, D. C.

¹ Jour. Immunol., 1922.

² Jour. Infect. Dis., 1922, 31, p. 148.

³ Centralbl. f. Bakteriologie, I. O., 1900, 27, p. 828.

Place 1,250 gm. of fresh, chopped, fat-free veal and 2 liters of distilled water in a pot and bring slowly to a boil, allowing it to simmer for 20 minutes, with occasional stirring. Strain through cotton flannel, cool and remove the fat. Place in a double boiler over a saturated brine bath and raise the temperature to about 60 C. Add 20 gm. of peptone (Difco used), 40 cc normal, fresh urine, 10 gm. NaCl, 40 cc glycerol and 36 gm. flaked agar. Allow this to boil for 45 minutes and then adjust the reaction to P_H 6.9, using 10% sodium carbonate, and boil for 30 minutes longer. Remove from the brine bath; make up loss from evaporation to 2 liters with distilled water. Filter through canton flannel and tube in 10 cc amounts. Autoclave at about 12 lbs. pressure for 10 minutes.

In preparing the plates, 5 cc of ascitic fluid, free from bile, and 0.5 cc of a 1:3,000 dilution of iodine-green (Grübler) are added to each tube of melted medium, just before pouring. The final reaction is generally about P_H 7.2. It should not be more alkaline than this.

If to be used in slants, the amount of agar is increased to 40 gm.

The following mediums contain a growth accessory principle. They are modifications of the "hormone" mediums, described by Huntoon,⁴ and have been found very useful for the purposes specified. As beef heart constitutes the meat element, they may be prepared at slight expense.

B. Ascitic, salt-free, 1.5% peptone, "vitamine" agar. This medium has been employed in connection with the isolation of the gonococcus.

Five hundred gm. of fresh, chopped beef heart, free from fat (the beef heart meat should preferably be obtained directly from the slaughter house), one whole egg and 1 liter of distilled water are placed in a double boiler over a free flame and the temperature maintained at 60 C., with constant stirring, for 5 minutes. Fifteen gm. of peptone (Difco) and 18 gm. of flaked agar are now added and the temperature raised until the mixture assumes a brownish color. The medium is then made slightly alkaline to litmus, using a 10% solution of sodium carbonate. It is next placed in a flask, or preferably a coffee pot, and heated at 100 C. in the Arnold steam sterilizer for 1 hour. The clot is then separated from the sides of the receptacle, and it is replaced in the sterilizer for another hour. It may be cleared by centrifuging or by straining through a fine wire mesh and then through glass wool. A clear medium may often be obtained if the meat residue is deposited on the glass wool in a funnel and the fluid portion allowed to percolate through several times. As Huntoon has stipulated, neither cloth, cotton nor any other material with absorptive properties should be used in clarifying the medium. After filtration, the reaction is brought to P_H 6.8. It is then reheated and tubed in 10 cc amounts. It may be sterilized in the autoclave at 12 lbs. pressure for 10 minutes, but fractional sterilization at 100 C. flowing steam is preferable. In preparing the plates, 5 cc of ascitic fluid is added to each tube of melted medium, just before pouring.

A modification of this medium, prepared without ascitic or other serous fluid and which has been found useful in obtaining growths for complement-fixation tests, is described elsewhere.¹

⁴ Jour. Infect. Dis., 1918, 23, p. 169.

C. A semi-solid agar with a growth accessory element (Huntoon). This medium, without the addition of ascitic fluid, has been found useful for maintaining our collection of stock strains. Even the most delicate strains of gonococcus have remained viable for 3 to 4 weeks without replanting, and the majority have lived for periods of 6 to 8 weeks or even longer, if kept at 37 C. The ingredients of this medium are the same as those stated in the formula of Huntoon.

Distilled water	1000 cc
Fresh chopped beef heart.....	500 gm.
Peptone	10 gm.
NaCl	5 gm.
One whole egg	

The same procedures are followed as in the preparation of medium B, and the final reaction is adjusted to P_H 6.8. This medium is tubed in about 7 cc amounts and is preferably sterilized in the Arnold sterilizer. The method of inoculating and the uses for this medium will be described. If ascitic fluid to the amount of about 1 cc per tube is added, the medium may be employed in connection with primary fishings of gonococcus colonies. It may also be used advantageously in the rejuvenation of delicate strains and in the recovery of old stock strains which have apparently died out.

CONDITIONS FAVORING MAXIMAL GROWTH OF THE GONOCOCCUS

Cole and Lloyd⁵ in their analysis of the cultural requirements of the gonococcus, determined that there are three factors of prime importance: first, the concentration of the hydrogen ions, or the reaction; second, the concentration of amino acids; and third, the presence of certain growth stimulating hormones or vitamins. The following paragraphs contain a report of our experiments on the effect of these various factors on the growth of this organism.

Reaction.—Cole and Lloyd reported the mean optimal reaction for growth as P_H 7.6, with a possible range of hydrogen-ion concentration between 6.5 and 8.6. They also stated that with relatively simple (unfavorable) mediums the reaction is of great importance, whereas in mediums containing a growth stimulating substance, a wide reaction range is compatible with good growth. Our findings agree in general with these statements, although our point of optimal growth is nearer neutrality. This lack of conformity as regards the optimal point may be due in part to the use of different standards for determining the H-ion concentration. We have used the colorimeter methods of Clark and Lubs,⁶ employing standard buffer solutions prepared according to their directions and which were, in turn, checked with solutions prepared in several other laboratories. They employed, on the other hand, the standards

⁵ Jour. Path. & Bacteriol., 1916-17, 21, p. 269.

⁶ Jour. Bacteriol., 1917, 2, p. 1.

of Cole and Onslow.⁷ Warden,⁸ several years ago, emphasized the importance of a slightly acid (+0.7 to 1.5 to phenolphthalein) reaction in mediums for the successful cultivation of the gonococcus. It is also our opinion that most investigators have employed mediums with a somewhat too alkaline reaction. Swartz⁹ has recently reported successful growth on an ascitic agar medium with a H-ion range from 6.5 to 8, if this organism is cultivated under conditions of partial oxygen tension. Erickson and Albert,¹⁰ using a beef testicular agar enriched with blood, found that the optimal reaction lay between P_H 7.4 and 7.6.

With our medium A, enriched with ascitic fluid, we have found that good growth may be obtained with a reaction ranging from about P_H 6.6 to 7.4, but if this medium is used without ascitic fluid the range is still more restricted toward the neutral point. With agar medium containing a growth accessory principle, a much greater latitude in reaction is permissible. The semisolid medium C has produced growth within a reaction range of from P_H 5.8 to 8.2, with an optimal zone between 6.4 and 7.7.

In table 1 are given both the degrees of growth at various H-ion concentrations with a stock strain of gonococcus, long under cultivation, and the remarkable period of viability observed in the lots of the medium with the more acid reaction. In this experiment 10 tubes of semisolid vitamine agar (C) of each reaction were seeded with gonococcus strain 33; the tubes were sealed with paraffin and kept at 36-37 C. From time to time replants were made into this medium C, which had been enriched with ascitic fluid, or on ascitic agar slants. With so many of the original cultures to choose from in testing for viability, it was possible to plant either from a hitherto unopened tube or, at least, from an undisturbed part of the original growth in a tube which had been opened before. As table 1 indicates, a tube with an initial reaction of P_H 6.3 still held viable organisms after the lapse of a year. With this particular strain, too, successful growths were obtained for far longer periods from the tubes on the acid side of neutrality than from those on the alkaline. It should be noted, however, that the initial reactions did not remain constant, but that the gonococcus growth caused a gradual change in the direction of alkalinity. On replanting from tubes which had been kept several months, the gonococcus colonies developed very slowly, no growth frequently appearing for 3 or 4 days. Cole and Lloyd⁵ have also reported long

⁷ Lancet, 1916, 2, p. 9.

⁸ Jour. Infect. Dis., 1913, 12, p. 93.

⁹ Jour. Urol., 1920, 4, p. 325.

¹⁰ Jour. Infect. Dis., 1922, 30, p. 269.

viability periods for gonococcus when growing in stab culture in their 2% tryptamine blood agar with an H- ion concentration of about 7.6, some of their strains surviving from 67 days to 5½ months. Morax¹¹ found that ascitic agar stab cultures might retain their viability for 6 months. Warden,⁷ using an artificial serum fluid medium, found that successful transplantation could be made after 100 days.

As the gonococcus surviving for one year without replanting was from an old stock strain and probably an unusually hardy one, a number of similar tests were conducted with recently isolated strains (table 2). As may be noted, the reaction of the medium, optimal for growth, was close to the neutral point. As regards the relation of reaction to viability, the tests with these strains yielded rather irregular results but, on the whole, at the periods of replanting a greater num-

TABLE 1
RELATION OF GROWTH TO VARIOUS REACTIONS AND THE RELATIVE PERIODS OF VIABILITY
AT VARIOUS REACTIONS. UNENRICHED SEMISOLID MEDIUM C

H-ion Con- centration of Medium	Degrees of Growth	Viability Tests: Replants in Weeks After Primary Cultures									
		3	4	5	9	16	22	29	42	46	52
5.4	—										
5.8	++	+	+	+	+	+	+	+	+	+	—
6.3	+++	+	+	+	+	+	+	+	+	+	+
6.8	+++	+	+	+	+	+	+	+	—	—	—
7.4	+++	+	+	+	—	—	—	—	—	—	—
7.8	++	+	—	—	—	—	—	—	—	—	—
8.4	—										

ber of cocci were found viable in the tubes with a slight degree of acidity than in those with the more alkaline reaction. A difference in the character of the growth in relation to the reaction was also noted in connection with all the strains. After about a week's incubation, the growth on the slightly acid to neutral medium became thick, soft and slightly buff-colored, whereas on the alkaline side of neutrality it was thinner, white and generally drier in appearance. These differences were also noted by Cole and Lloyd. They observed, too, that autolysis apparently proceeds more rapidly on mediums more alkaline than that considered the mean optimum. Our observations add confirmation to this conclusion.

As to the relation of the reaction of the medium to viability, the tests with these strains yielded rather irregular results but, on the

¹¹ Ann. de l'Inst. Pasteur, 1918, 32, p. 471.

whole, at the times of replanting a greater number of cocci were found viable in the slightly acid tubes than in those with the more alkaline reaction.

These periods of viability (table 2), which are by no means the limits for even these delicate strains, contrast strongly with those obtained by others on slants of solid medium. Swartz¹⁰ has reported a viability period of 7 to 10 days on slants of his ascitic agar medium and Cook and Stafford¹² of only 8 days with slants of their enriched

TABLE 2

RELATION OF VARIOUS REACTIONS TO THE GROWTH OF RECENTLY ISOLATED GONOCOCCUS STRAINS AND RELATION OF REACTION TO VIABILITY *

H-ion Con- centration of Medium	Relative Degrees of Growth, Gonococcus Strains								Tests for Viability: Colony Development from One Loop						
	28	63	66	67	70	74	75	76	28 30 Days	66 21 Days	70 21 Days	74 45 Days	75 26 Days	76 26 Days	
6.0	1	1	1	2	0	0	2	0	Numerous	Fairly numerous	—	—	∞	—	
6.4	3	3	2	2	0	1	3	1	∞	0	—	Few	∞	∞	
6.8	3	3	2	2	2	1	3	1	0	0	Very numerous	Numerous	Fairly numerous	∞	
7.3	3	2	3	2	2	1	2	2	0	0	Very numerous	0	∞	Few	
7.7	2	1	2	2	1	1	2	1	0	Fairly numerous	Numerous	Few	Numerous	Numerous	
8.0	2	...	1	1	0	0	1	1	Fairly numerous	0	—	—	Fairly numerous	Fairly numerous	
8.2	1	1	1	1	0	0	0	0	0	0	—	—	—	—	
8.6	0	0	0	0	—	—	—	—	—	

Strains 75, 76: third generation from isolation.

Strain 74: fourth generation from isolation.

Strains 63, 66, 70: fifth generation from isolation.

Strain 28: very delicate strain.

* Primary plants were made on semisolid agar (C) and tubes sealed with paraffin. Replants for viability were made on ascitic agar slants, using one uniform loop of the primary culture.

testicular agar, and no recoverable growth after 3 days with stab cultures. Hermanies,¹³ however, using salt-free ascitic agar slants, about neutral to litmus, found that some of his strains would live for weeks or even months without transplanting.

Amino Acids.—Cole and Lloyd have emphasized the important influence of amino acids on the development of the gonococcus, especially in the presence of "growth hormones." Their amino acids were obtained by a tryptic digestion of casein and the product was desig-

¹² Jour. Infect. Dis., 1921, 29, p. 561.

¹³ Ibid., 1921, 28, p. 133.

nated "tryptamine." In experiments bearing on this point we have used preparations from liver, casein and beef in which the amino acids constituted from 65 to 75% of the total nitrogen.¹⁴ These products were used in 1% amounts in place of peptone in preparing the semi-solid medium C, and the growth and viability on these mediums were compared with that on the medium prepared with peptone, but otherwise identical. It was not found that these mediums, unusually rich in amino acids, produced a greater or a quicker growth; in fact, with some strains the results were inferior to those obtained with the peptone medium. Accordingly it was concluded that the peptone¹⁵ used, especially if employed in a concentration of 1.5%, contained sufficient amino acids to effect the optimal development of the gonococcus. In fact, M'Leod and Wyon¹⁶ recently reported that high concentrations of amino acids readily inhibit the growth of such organisms as pneumococci, meningococci and hemolytic streptococci.

Growth Stimulating Substances.—A third factor, considered by Cole and Lloyd of primary importance in the cultivation of the gonococcus, are two different growth stimulating substances, present in fresh blood and other materials, which were designated as "growth hormones." The substance of importance in inducing initial growth was considered a derivative of red blood cells and was shown to be readily absorbable by colloidal substances, such as agar and gelatin, and also by materials ordinarily used in clarifying mediums. The second substance, present in animal and plant tissues, was relatively non-absorbable, and was thought to stimulate luxuriant secondary growth. The first substance was considered to be of the same nature as vitamins or hormones because of the ease with which it was absorbed. As the term, vitamin, would seem to be a rather more appropriate designation for the substance stimulating primary growth, it will be employed hereafter.

Huntoon,⁴ utilizing these principles of Cole and Lloyd, has described simpler methods for preparing these vitamin mediums. Instead of blood, beef heart or steak was employed with the idea that these tissues would provide sufficient of the growth accessory substances, especially when fortified with a whole egg. We have found mediums of this type useful in our work with the gonococcus, and have followed

¹⁴ Prepared by the Arlington Chemical Co.

¹⁵ Different lots of this peptone have varied somewhat in efficiency as growth producers, but only one sample was encountered which could not be used.

¹⁶ Jour. Path. & Bacteriol., 1921, 24, p. 205.

Huntoon's methods with certain modifications in the adjustment of the reaction, the amount of peptone and the use of salt. Beef hearts have been used entirely as the meat base, and it has been deemed important to obtain them as fresh as possible. In all probability the primary growth stimulating substance is derived mostly from the blood content of the meat and in minor degree from the tissues.

The desirability of limiting the degree and duration of heating in the preparation of mediums of this type was emphasized by Huntoon on the supposition that the growth stimulating substances are to some degree heat labile. Thjotta and Avery,¹⁷ in a careful analysis of the properties of the growth accessory substances essential for the cultivation of the hemophilic bacilli, determined that their V factor, derivable from blood and from yeast and other vegetable cells, was not impaired appreciably by boiling for 10 minutes, but their potency was greatly impaired by exposure in the autoclave to 120 C. for 30 minutes. In the preparation of these vitamine mediums for the gonococcus, it has been necessary to apply a boiling temperature for far longer periods than that specified by Thjotta and Avery as a safe limit. There can be no doubt, however, that the growth stimulating substances for the gonococcus survive this rather prolonged exposure to 100 C. in sufficient amount and degree to influence its development very favorably. That this is the case is shown by the experiments of Cole and Lloyd and also by our own results. The question arose, however, as to whether these growth accessory substances would resist the higher temperature of the autoclave. In order to test the effect of this heat factor a number of the most delicate strains in our collection were seeded on a particular lot of the semisolid vitamine agar (C) the several portions of which were subjected to the various degrees and durations of heating, as shown in table 3. Under column B of this table there is also demonstrated the adsorptive action of flannel and cotton on these accessory growth substances. Thjotta and Avery also found that their V factor, essential to the growth of *B. influenzae*, was readily adsorbed by bone charcoal.

The results reported in table 3 indicate that the growth stimulating substance in this medium is slightly impaired by the autoclave temperature of 120 C. for 5 minutes and seriously injured by a temperature of 120 C. for 30 minutes, and also by prolonged heating in the Arnold sterilizer. In estimating the degree of injury the results with lots A

¹⁷ Jour. Exper. Med., 1921, 34, p. 97.

and B serve as good comparates; in the former the medium exhibits its maximal efficiency, whereas from the latter the growth stimulating element has been in large degree removed by filtration through absorbing substances.

Moisture.—It has long been realized that the gonococcus will not grow well on solid mediums from which the initial moisture has largely dried out. Only recently, however, has the importance of a moist air in the incubator been appreciated. Jenkins¹⁸ has reported that a moist atmosphere, obtained by placing a large dish of water in the incubator, materially accelerates the growth of the gonococcus. Cook and

TABLE 3

THE EFFECT OF FILTRATION AND OF DIFFERENT DEGREES AND PERIODS OF HEATING ON THE GROWTH STIMULATING PRINCIPLE OF AGAR MEDIUM C *

Gonococcus Strains	A		B		C		D		E	
	3 Days	12 Days	3 Days	12 Days	3 Days	12 Days	3 Days	12 Days	3 Days	12 Days
3	++	++	—	—	—	—	—	—	—	—
40	—	++	—	—	—	—	—	++	—	—
54	++++	++++	—	—	+	++	+++	++++	++	++
69	+	+	—	—	—	—	—	—	—	—
27	+++	+++	—	—	++	+++	+++	+++	+	++
41	++++	++++	++	++++	+	++	++	++++	++	++++
34	++++	++++	++++	++++	++++	++++	++++	++++	++	++++

+, ++, +++, ++++ indicate degrees of growth.

* Different portions of one lot of this semisolid medium treated as follows:

Lot A: Filtered through glass wool and heated at 100 C. in the Arnold Sterilizer for 20 minutes on 3 consecutive days.

Lot B: Filtered twice through flannel and absorbent cotton and sterilized as A.

Lot C: Filtered through glass wool and heated in the Arnold sterilizer for 2 hours on each of 3 consecutive days.

Lot D: Filtered as for C and heated in the autoclave at 120 C. for 5 minutes.

Lot E: Filtered as for C, and heated in the autoclave at 120 C. for 30 minutes.

The H-ion concentrations of these several lots after final sterilization ranged from PH 6.5 to 7.0 (within the zone of optimum growth).

Tubes of these several lots were seeded with one loop each of a saline suspension (about 3 billion strength) of gonococci. Strains 3, 40, 54, 69, 27 were of the delicate type.

Stafford¹² found that the best growths resulted when the culture tubes were placed in closed jars containing water and that placing a pan of water in the incubator was not sufficient. It has been our experience that a large bowl or pan of water (about 10 inches in diameter) provides enough moisture for the ordinary sized incubator. There can be no doubt that the presence of such a moist atmosphere is a matter of prime importance, especially in obtaining primary growth from infected material. It acts as a marked accelerator of growth; colonies appear several hours earlier on the plates and in greater number than

¹⁸ Jour. Bacteriol. & Path., 1921, 24, p. 160.

when incubated without special provision for moisture; in fact, there may be no growth at all in the presence of the ordinary dry air of the incubator.

As regards the physical state of solid medium, we have found that the best results are obtained when it is moderately moist and firm. For slants, after the proper amount of ascitic fluid has been added, the agar content should amount to about 1.5% and for plates rather less than that. Hall¹⁹ has advised a hard, firm surface, free from excessive moisture, for optimal growth, but we believe that a very firm surface, such as is obtained with 2.5% agar, is decidedly unfavorable. Ascitic agar slants should be prepared the day before they are to be used and should generally be allowed to drain for an hour in the incubator before seeding. Culture plates should be poured several hours before they are to be streaked, or, if the medium has its full quota of moisture, may be prepared the day before.

Reduced Oxygen Tension.—Within recent years a number of investigators have advocated a reduced oxygen tension atmosphere as especially favorable for the growth of the gonococcus. This reduction in tension has been effected in various ways; through heating the air in the culture tube and closing tightly with a rubber stopper (Ruediger,²⁰ Swartz⁹); through a partial exhaustion of the air (Swartz⁹); through exposure to a CO₂ atmosphere (Chapin²¹); through the use of a bacterial culture with oxygen reducing properties, such as *B. subtilis* (Wherry and Oliver,²² Herrold,²³ Hermanies¹³). From time to time we have tested these various procedures, but the results have been in no way superior to those obtained on the same medium exposed to moist air of normal pressure. The gonococcus in fact does not behave like an organism with a predilection for reduced oxygen tension. In stab cultures the growth in the stab is, at best, very feeble; in fact, in our semisolid medium it occurs exclusively on and immediately below the surface. It is worthy of note that all the procedures utilized for maintaining a reduced oxygen tension also tend to retain moisture in the medium or in the air, which is in itself an important factor in obtaining the maximal growth for the gonococcus.

¹⁹ Jour. Bacteriol., 1916, 1, p. 343.

²⁰ Jour. Infect. Dis., 1919, 24, p. 376.

²¹ Ibid., 1918, 23, p. 342.

²² Ibid., 1916, 19, p. 288.

²³ Jour. Am. Med. Assn., 1921, 76, p. 225.

Cook and Stafford¹¹ and Erickson and Albert⁹ have also recently reported that a reduced oxygen tension does not favor the growth of the gonococcus.

THE PRIMARY ISOLATION OF THE GONOCOCCUS

As it was seldom feasible to inoculate the culture plates immediately after the infected material had been obtained from gonorrheal cases, the following method was devised whereby a delay of several hours might elapse with a minimal risk of loss of viability on the part of the gonococci.

One c.c portions of a mixture of 2 parts of semisolid "vitamine" agar (C) and 1 part of ascitic fluid are placed in narrow test tubes (6" x 1/2"). This mixture is semifluid in consistency. After the swab has been infected with the gonorrheal discharge it is placed in a tube containing this medium, care being taken that the swab becomes well moistened with it, and is left there. This tube is then placed under the clothing; preferably next to the skin. It is advisable, although not essential, to warm the medium slightly just before introducing the swab. On reaching the laboratory the tubes containing the swabs are placed at once in the incubator. The plates, which should be slightly warmed, may be seeded then or the tubes may be left there for 3 or 4 hours before this is done. Within this period the gonococci apparently do not die out at all; in fact, they may begin to increase in numbers. The plating, however, should not be delayed so long that the contaminating bacteria have an opportunity to overgrow the gonococci. Even at room temperature gonococci in pus deposited on the surface of an ascitic semisolid agar tube and kept in a dimly lighted place have remained viable for a surprisingly long time. In 2 such experiments living gonococci were found after 48 hours in pus left under such conditions, although their numbers were reduced to a fraction of 1% of that originally present. After 3 days all had died. These specimens of pus were obtained from cases of acute urethritis in males and contained large numbers of gonococci. The tests offer a fair criterion of the probable maximal infectious period of such gonorrheal discharges, when kept moist at ordinary temperatures and removed from contact with strong light.

In taking specimens from cases of urethritis in males it was not found necessary to clean or treat the meatus in any way, especially if the discharge was taken up with a platinum loop. Pus from joint

cases should be distributed among 3 or 4 tubes of the semifluid ascitic agar, described in the foregoing paragraph, and plates seeded from them at once and after incubation for 24 to 48 hours.

In seeding plates the swabs are applied to only about one-fourth of the surface area of the medium. The swab should be rolled so that all parts come in contact with the medium. With a platinum loop the other three-fourths of the plate surface are now seeded by streaking from the part to which the swab was applied. In that way a proper distribution of colonies is likely to be obtained on some part of the plate. Before a second plate is seeded the swab should be reintroduced into the tube containing inoculated semifluid ascitic agar and so on for each plate.

In the preparation of plates for gonococcus isolation we have employed both A and B agar mediums, using with each 2 parts of the medium and 1 part of ascitic fluid. As is well known, some samples of ascitic fluid are unsuited for gonococcus culture. The lots used successfully were free from bile and had a specific gravity of 1.010 or higher. As will be explained presently, certain dyes have been used at times in conjunction with these mediums.

Colony Characteristics.—Medium A with ascitic fluid produces a colony which is rather different from that regarded as typical for the gonococcus. These colonies after 48 hours' incubation are only semi-translucent in texture and have a raised, even, or, at most, a slightly indented edge. They stand out prominently from the surface of the medium. The centers are somewhat thickened and exhibit few or numerous light colored granules. The edges are clear and homogeneous. By transmitted light they have a rather characteristic light fawn color with a suggestion of a greenish tinge. In consistency they are somewhat viscid or pasty. On primary isolation the colonies are generally just visible to the naked eye after 24 hours' incubation and are not characteristic in appearance. After 48 hours, however, they may attain a diameter of 1 to 3 mm. and are easily identified. They often continue to grow for about 10 days when they may reach a diameter of 8 mm. The older colonies have heaped up centers with thin spreading edges. One of the advantages of this medium is the unusual retention of viability even in the primary growth; successful replants have been made from such colonies up to 10 days. The gonococcus colonies are also distinctive in appearance and after a little experience are hardly to be mistaken.

The medium B with its growth accessory element may be used to advantage in conjunction with medium A. This plate medium may bring to development over 10 times as many gonococcus colonies as medium A. The colonies also develop more rapidly and may be visible in 18 hours. After 24 hours' incubation they frequently attain a diameter of about 1 mm. These colonies are colorless and translucent, showing a light smoky tinge by transmitted light. They may show heaped up centers or a flat surface. The edge may be very thin and slightly crenated or may be raised, well defined and smooth. The well isolated colonies exhibit the latter appearance and under low magnification show numerous light colored crumbs occupying the entire colony except the outer periphery. Whereas fishings from the A medium plates may be delayed for 3 or 4 days, the gonococcus-like colonies on the B plates should be fished by the second day as the rapid growth seems to be correlated with speedy disintegration. Among other organisms forming a colony similar to the one described in the foregoing, we have noted a gram-positive diplococcus and a small gram-negative coccoid bacillus.

Fishing of Colonies.—Fishings of colonies for the most part have been made into the ascitic semisolid medium (C). The growth on the surface of this medium is so characteristic as to be almost diagnostic in itself. The fished colony is seeded on and by short stabs all over the surface of this medium, which has the advantage of being not only favorable for the primary growth but also for the prolonged viability of the gonococcus. Of such original plants from the plates of 6 cases, the tubes of which had been sealed with paraffin, all were found alive after 50 days at 36 C. and 3 of them after 80 to 90 days. With this medium, then, there is no danger of losing a strain on first isolation and no need of making such frequent replants as has been considered necessary heretofore. It would seem entirely probable that this medium would prove as well adapted for fishing meningococcus colonies. After the first generation of the gonococcus on this ascitic semisolid medium, it is generally possible to transplant successfully to the medium without ascitic fluid; in a number of cases fishings have been made directly to the semisolid medium unenriched with ascitic fluid and growth obtained. Most strains, however, thrive only when the fishing is made to the enriched medium.

Selective Dyes.—Certain dyes, especially gentian violet and brilliant green, have proved so useful in the preparation of selective mediums for

various bacterial types that it was hoped some one might be found of service in the isolation of the gonococcus. A considerable number of dyes were tested and of these, iodine-green and methyl violet gave the more promising results. In most instances the test material consisted of swabbings from mild chronic vulvovaginitis cases in children. Frequently in such cases other types of bacteria are so numerous that isolation of pure cultures of gonococcus is a matter of great difficulty. In the experiments with iodine-green (Grübler), the dye was diluted 1:2,000 or 1:3,000 and 0.5 to 1 c c added to 15 c c of the ascitic agar medium A. It was observed that this dye, at the dilution used, exercised its inhibitory effect only when glycerol was present in the medium. Under these conditions the dye tends to suppress some types of gram-positive cocci—not streptococci—and certain diphtheroids. Colonies of staphylococci and diphtheroids which succeed in developing on the plates are tinted green, whereas the gonococcus colonies are either uncolored or show only a faint greenish tinge when viewed against a dark background. This dye, in the dilutions used, seemed actually to stimulate the growth of gonococcus as these colonies tend to become larger on plates containing the dye than on the control plates. In fact, the use of this dye in connection with this plating medium was finally adopted more on this account than because of its inhibitory effect on other bacteria, which, indeed, is rather slight.

Of the triphenylmethane dyes, gentian violet and methyl violet were the only ones tested extensively. Gentian violet suppresses effectively the great majority of gram-positive bacteria, but unfortunately gonococcus, although a gram-negative organism, is also highly susceptible to its toxic action. The strongest dilution which could be used with any degree of success was a final one of 1:1,200,000 (1 c c of a 1:80,000 dye dilution in 15 c c of medium B). Although most gonococcus strains exhibit a slightly greater tolerance to the bacteriostatic action of gentian violet than do the majority of gram-positive cocci and diphtheroids, the margin of difference is so slight that the use of this dye as a selective agent offers slight promise of success. Occasionally, when using gentian violet medium in making cultures from gonorrheal discharge, we have obtained a plate showing a nearly pure culture of gonococcus with a marked suppression of the gram-positive organisms in the specimen, but comparison with the control plate would indicate that only a very small percentage of the viable gonococci in the pus had developed

into colonies. On the gentian violet plates, also, cultures did not become visible to the naked eye until the second or third day of incubation. Cook and Stafford¹² have recently reported on the use of gentian violet and also other triphenylmethane dyes as ingredients of a selective medium for the gonococcus. Gentian violet was used in connection with testicular or chocolate blood testicular agar in a dilution of 1:500,000. The results obtained, however, in the application of this medium to clinical diagnosis were not promising.

As is well known, Churchman²⁴ has advocated the use of gentian violet in the local treatment of cases of purulent arthritis including the gonorrheal type. In discussing the results obtained, he observed that as the gonococcus is a gram-negative organism it might be expected to be relatively unaffected by gentian violet, but he was unable to settle this point experimentally. Our experiments would seem to indicate, however, that the gonococcus exhibits only a slightly lower degree of susceptibility to the bacteriostatic action of gentian violet than do the gram-positive cocci and hence should be classified as a "gentian violet positive organism."

In connection with the isolation of the gonococcus, methyl violet has yielded somewhat better results in our hands than has gentian violet. In our more successful experiments this dye has been diluted to about 1:125,000 and 1 c c added to 15 c c of the ascitic agar medium B. A stronger dilution than this tends to be too inhibitory of the development of gonococci. Staphylococci have been quite effectively suppressed as have also some troublesome types of diphtheroids and spore-bearing bacteria. On the other hand, streptococci and gram-negative bacilli are not affected at all. This dye, accordingly, is only to a limited degree selective for the gonococcus, but we have found at times that its presence in the medium has permitted the isolation of this organism when the control plates were covered with growth of other bacterial types. The advantages and limitations in the use of this methyl violet medium may be estimated from the results reported in a following article.²⁵ As will be observed, it may be used to some advantage in connection with other plating mediums but dependence should not be placed on it alone. This dye has been used only with the ascitic "vitamine" agar B. The optimal dilution should be determined for each

²⁴ *Ibid.*, 1920, 75, p. 583.

²⁵ *Jour. Infect. Dis.*, 1922, 31, p. 148.

sample of dye used. Erickson and Albert⁹ have recently reported that of various violet and green dyes tested, methyl violet was the most effective for isolation of the gonococcus.

Reaction.—The final reaction of these plating mediums has always been close to P_H 7.2.

CRITERIA FOR THE IDENTIFICATION OF THE GONOCOCCUS

If the material for culture has been obtained from the genito-urinary tract, we have found that the type of colony formation together with morphology and staining reaction is an almost infallible guide to the identification of the gonococcus. As has been mentioned, only one or two types of bacteria from this locality form colonies closely resembling that of the gonococcus and in no case do these bacteria bear a resemblance morphologically to this organism. Cultures from several hundred cases of gonorrhea have never shown any colonies of *M. catarrhalis* or any other diplococcus morphologically resembling the gonococcus, with its typical picture of a mixture of well-staining, biscuit-form diplococci and swollen, more or less completely autolyzed, irregularly staining cocci and diplococci. Although this is the case, we have not depended on these points alone for identification.

Inability to grow on unenriched medium during the first few generations has been generally accepted as an important diagnostic test. The great majority of gonococcus strains will conform to this requirement, but occasionally we have encountered undoubted gonococcus strains which would grow slowly on ordinary glycerol, beef infusion, peptone agar slants with a reaction of P_H 7.4, on planting from the second or third generation of subcultures. These strains were mostly isolated from vulvovaginitis infections in children. Some years ago Wollstein²⁶ reported that strains from such sources grew readily on plain agar. This capacity, however, may not be considered as a cultural feature differentiating strains causing infantile vulvovaginitis from those concerned in the gonorrhea of adults, for we have isolated from these children's cases strains of a type which was very delicate and difficult to cultivate. No gonococcus strain, of course, will grow at room temperature on even the most favorable type of medium. Serologic tests, such as agglutination and agglutinin absorptions, cannot be depended on as a certain guide to identification, as has been explained elsewhere. That fermentation tests are of great value in the differentiation of the

²⁶ Jour. Exper. Med., 1907, 9, p. 588.

gram-negative diplococci has been demonstrated by Elser and Huntoon and others. As will be shown presently, our tests with over 80 strains proves that the gonococcus is capable of splitting glucose alone and is thus to be differentiated from its nearest relative, the maltose fermenting meningococcus.

The characteristics distinguishing the gonococcus from other similar organisms may then be summarized as follows: appearance of the colony, reaction to the gram stain and morphology, inability to grow at room temperature and typical fermentation reactions.

FERMENTATION TESTS

Elser and Huntoon,²⁷ in an extensive series of fermentation tests with different species of gram-negative diplococci capable of a parasitic existence within the human body, found that of the 10 carbohydrates employed in the tests, the gonococcus was the only representative of this group which fermented glucose. In this conclusion they confirmed the earlier finding of Rohe²⁸ that of glucose, maltose and levulose, the gonococcus splits glucose alone. Within recent years there has been a general agreement that the gonococcus does not ferment maltose and by that fact may be differentiated from the meningococcus. As to the action of gonococcus on galactose, however, there is no such uniformity of opinion. The earlier observers, Dunn and Gordon,²⁹ Arkwright³⁰ and Sherman and Ritchie,³¹ all report the fermentation of galactose by gonococcus. Elser and Huntoon, however, obtained negative results in tests with 15 strains of this organism. More recently Cole and Lloyd⁵ reported that galactose was fermented by the gonococcus but that the acidity was not as great as when glucose was the test sugar. As a probable explanation of the discrepancies in these findings certain experiments of Elser and Huntoon with galactose may well be cited. They found that with intermittent streaming steam sterilization for the customary time periods, galactose and also levulose in a menstruum containing very small amounts of free alkali were likely to be hydrolyzed with the production of acidity, and that this change continued in some instances when the medium was exposed to incubator temperature. However, by using glassware devoid of free alkali and by sterilizing the

²⁷ Jour. Med. Research, 1909, 20, p. 377.

²⁸ Centralbl. f. Bakteriöl., O., 1908, 46, p. 645.

²⁹ Brit. Med. Jour., 1905, 2, p. 421.

³⁰ Jour. Hyg., 1907, 7, p. 145.

³¹ Jour. Path. & Bacteriol., 1908, 12, p. 456.

sugars separately through exposure of the distilled water solutions to live steam for 10 minutes, they demonstrated that these hydrolytic changes may be avoided.

As a base for our fermentation tests we have employed a sugar-free broth plus ascitic fluid. Beef infusion was made sugar-free by planting with *B. coli* and incubating for 24 hours. To the filtrate was added 1% peptone and 0.5% NaCl. After adjusting the reaction to P_H 7.0, the medium was tubed in 5 c c amounts and autoclaved. One c c of ascitic fluid was then added to each tube. The ascitic fluid used had been in cold storage for over 4 months, a period sufficiently long to permit the complete disappearance through hydrolysis of any fermentable carbohydrate which it might have contained. 12% solution of the sugar in distilled water was exposed to flowing steam at 100 C. for 12 minutes and 0.5 c c was added to each tube of the ascitic broth. These tubes of medium were then incubated for 3 days at 37 C. as a test for sterility. The consistent results obtained with levulose, galactose and maltose indicate that these relatively unstable sugars were not injured by the degree and period of heating applied in sterilization.

In these comparative fermentation tests we have used a fluid medium because it permitted a determination of the exact degree of change in reaction through the use of colorimetric methods. As a routine method, however, a solid or semisolid medium to which an indicator has been added is to be preferred as the growth of the gonococcus is much more rapid on such a medium and a reading may be made within 24 to 48 hours. A medium of this type, which has given satisfactory results, is described in a following paragraph. As is indicated in table 4, reaction readings for these gonococcus strains in the various fluid sugar mediums were made after 7 days' incubation. The H-ion concentrations were determined through the use of bromthymol-blue or phenol red and comparisons with their respective scales as the conditions called for.

In this tabulation of fermentations (table 4) the results with 60 gonococcus strains are given in detail. In addition 25 gonococcus strains isolated from cases of mild chronic gonorrhea in women were tested on glucose and maltose, making a total of 85 strains. These strains had been isolated from a great variety of clinical conditions, including acute and chronic urethritis in males, vulvovaginitis in children, arthritis cases, septicemias and cervix uteri infections. They also represented widely separated geographical localities: many parts of this country and such foreign countries as Mexico, England, France, Bel-

TABLE 4
FERMENTATION TESTS WITH 60 GONOCOCCUS STRAINS *

Gonococcus Strains	Glucose P _H	Galactose P _H	Maltose P _H	Levulose P _H
1.....	6.5	7.1	7.3	7.4
2.....	6.6	7.2	7.3	7.4
3.....	6.4	7.0	7.1	7.5
4.....	6.0	7.1	7.3	7.4
5.....	6.3	7.2	7.5	7.6
6.....	6.4	7.2	7.3	7.3
7.....	6.2	7.2	7.8	7.8
8.....	6.5	7.2	7.3	7.4
10.....	6.4	7.0	7.3	
11.....	6.2	7.2	7.3	7.4
12.....	6.2	7.4	7.5	7.3
13.....	6.0	7.0	7.2	
14.....	6.4	7.5	7.5	7.8
15.....	6.2	7.4	7.5	7.4
16.....	6.5	7.0	7.2	7.1
17.....	6.2	7.5	7.5	7.6
18.....	6.2	7.3	7.6	7.3
19.....	6.7	7.5	7.5	8.2
20.....	6.2	7.0	7.2	7.3
21.....	6.2	7.1	8.0	7.6
22.....	6.6	7.1	7.3	7.3
23.....	6.4	7.6	8.0	7.3
24.....	6.5	7.1	7.2	7.3
25.....	6.1	7.2	7.5	7.4
26.....	6.0	7.3	7.2	
27.....	6.3	7.1	7.4	7.3
28.....	6.2	7.1	7.6	7.3
29.....	6.2	7.5	7.3	7.3
30.....	6.0	7.1	8.0	
31.....	6.2	7.8	8.1	
32.....	6.0	7.2	7.2	
33.....	6.0	7.1	7.2	
34.....	6.0	7.6	7.2	
35.....	6.7	7.4	8.0	
36.....	7.2	8.0	7.4	
37.....	6.2	7.2	8.0	
38.....	6.2	7.2	7.2	
39.....	6.2	7.3	7.3	
40.....	6.5	6.9	7.1	
41.....	6.2	7.2	7.4	
42.....	6.2	7.2	8.2	
43.....	6.3	7.1	7.4	
44.....	6.2	7.3	7.8	
45.....	6.3	7.3	7.3	
46.....	6.5	7.2	7.8	
47.....	6.5	7.2	7.3	
48.....	6.2	...	7.3	
49.....	6.2	7.3	7.3	
50.....	6.2	7.1	7.4	
51.....	6.4	7.2	8.0	
52.....	6.0	7.3	7.2	
53.....	6.2	7.1	7.2	
54.....	6.2	7.3	7.4	
55.....	6.2	7.4	7.8	
56.....	6.5	7.4	7.9	
57.....	6.3	7.2	7.2	
58.....	6.5	7.4	7.8	
59.....	6.4	7.1	7.3	
60.....	6.7	7.4	7.1	
61.....	6.2	7.4	7.8	
Control: meningococcus.....	5.9	7.3	5.8	
Control 1 (sugar mediums uninoculated and incubated).....	7.2	7.1	7.2	7.4
Control 2 (medium without sugar inocu- lated with gonococcus and incubated).	7.2			

* All tests for H-ion concentrations were made after 7 days' incubation.

gium, Germany and Egypt. As may be noted, with one exception these strains all produced a definite acidity in the glucose broth medium. The strain failing to split this sugar was an old stock culture (strain "G," redesignated 36). This strain grew very well in the medium but in repeated tests failed to attack the sugar at all. It would seem likely that we have here an instance of repressed or lost function rather than an exception to the general rule that the gonococcus ferments glucose. The strains differed considerably in the amount of acid produced but this variation was not definitely correlated with either the age of the strain or the amount of growth; some of the strains producing only a slight amount of acid were among the most vigorous growers.

As is indicated in table 4, none of the strains tested fermented galactose, maltose or levulose. The results with galactose are of rather more academic than practical interest as, according to the results of Elser and Huntoon, none of the gram-negative diplococci, which bear a close resemblance to the gonococcus, split this sugar. On the other hand, the uniform absence of action on maltose adds confirmation to the value of this sugar for the differentiation of the gonococcus from the meningococcus. The meningococcus strain, used as a control, acted promptly on this maltose medium, producing a marked degree of acidity in 48 hours. No recent observer has definitely claimed that the gonococcus may ferment maltose. Hermanies¹² recently reported in reference to a large number of gonococcus strains that "practically none of them fermented maltose," the exceptions apparently being 2 strains giving rise to a slight initial acidity which gave way after 48 hours to alkalinity. Wollstein,²⁰ in 1907, claimed that 10 strains of gonococcus from cases of vulvovaginitis in infants, all fermented maltose. Our tests, however, have indicated that such infantile strains do not differ in their fermentations from strains isolated from adults; 8 such strains (48, 54, 55, 56, 57, 58, 60, 61) gave positive results with glucose and negative results with galactose and maltose.

In the galactose and levulose medium, and to a rather more marked extent in the maltose medium, some degree of alkalinity was produced by the majority of the strains after 7 days' incubation. This degree of alkalinity was quite definitely linked with the vigor of the growth.

Our results have shown definitely that the presence of a sugar fermentable by the gonococcus in a medium does not enhance its growth, for fully as vigorous growth occurred in this fluid medium without any sugar or with the nonfermentable sugars as in the glucose tubes. The

same finding also held true for solid mediums. We can thus confirm the conclusion of Cole and Lloyd⁵ that the addition of glucose to mediums prepared for gonococcus culture is not desirable.

A few of our gonococcus strains grew so poorly in this fluid medium that it could not be used for determining their fermentative activities. This medium is also not well adapted for diagnostic tests as the necessary incubation period covers several days. We have found that a sure growth with a definite reading after about 24 hours' incubation may be obtained by using a semisolid agar medium to which brom-thymol-blue has been added as an indicator. This medium is prepared with meat-infusion, sugar-free, 1.5% peptone broth to which $\frac{2}{3}\%$ agar is added. To this semisolid agar, adjusted to P_H 7.0, is added brom-thymol-blue in an amount sufficient to give a fairly deep color. It is tubed in 5 c c amounts, sterilized, and the ascitic fluid and sugars added as advised for the fluid medium. The gonococcus growing on the surface of this unslanted medium causes, within 24 to 48 hours in the presence of glucose, a definite change in color from bluish green to yellow in the medium immediately below the growth. With some strains the change to yellow is evident within 18 hours. In the presence of maltose or other nonfermentable sugar the color either remains unchanged or a bluish tinge develops. This dye indicator does not inhibit the growth of the most delicate strains.

MAINTENANCE OF STOCK STRAINS OF GONOCOCCUS

The maintenance of a large collection of gonococcus strains on ascitic agar or blood agar slants is a troublesome matter and necessitates their being transplanted at intervals of a week or less, in fact some investigators⁹ have advised daily replanting of stock cultures. We have found that these difficulties may be in large measure eliminated by the use of the semisolid medium C, which contains a growth accessory principle and is prepared, with slight modifications, according to the method of Huntoon. In using this medium the gonococcus growth is seeded into the upper one-fourth inch or so of the unslanted agar, which has a H-ion concentration of about 6.8. One of the great advantages of this medium is that it is sterilizable, and one may thus avoid the contaminations which are so prone to occur if ascitic fluid or other nonsterilizable albuminous fluids are used; also this medium does not dry out, and hence favors the development of a thick moist growth with most gonococcus strains. With stab cultures into this medium, the

growth is limited to about 4 mm. below the surface, none occurring in the depths of the stab. Huntoon also recommended a semisolid medium of this type for the preservation of stock cultures, reporting a viability period of 3 months for the meningococcus and 2 months for the gonococcus.

The viability factor with this medium has already been discussed from the standpoint of various H-ion concentrations. As was pointed out in that connection, some strains remain viable in this medium for periods ranging from several months to one year; other strains of the more delicate type may not give successful replants in this unenriched medium longer than one month. As a matter of routine, accordingly, the stock strains of the gonococcus have been replanted about every three weeks. Any strain failing to grow could always be recovered by the use of this medium enriched with ascitic fluid (see p. 126). These exceptionally delicate strains exhibited on first isolation the common characteristics of producing an unusually viscid growth with rapid autolysis of the cocci. On prolonged cultivation, however, most of them tended to lose these characteristics and to become much more hardy.

Young growths of gonococcus on this medium placed at room or icebox temperature tended to die out quickly. The limit of viability at both these temperatures ranged from less than 3 days to not more than 6 days. Hermanies, on the other hand, using ascitic agar slants, found his stock strains of gonococcus uniformly viable after 8 to 10 days at room temperature, and some remained so for periods up to several months. Cook and Stafford, using an unenriched testicular agar, obtained a viability period limited to 8 days, regardless of whether the cultures were kept at incubator, room or icebox temperatures. We have found the optimal temperature to be between 36 and 37.5 C., although continued viability is possible with temperatures up to 38.5 C.

SUMMARY

For the optimal growth of the gonococcus the reaction of the medium should be set close to the point of absolute neutrality; between P_H 6.8 and 7.4. The reaction range, however, compatible with growth on a semisolid medium containing a growth accessory factor was found to extend from P_H 5.8 to 8.2.

The relation of viability to the reaction of a medium was studied. A slightly acid reaction was found, on the whole, more favorable than

a slightly alkaline reaction. The remarkable retention of viability for one year was noted in reference to one strain seeded on a semisolid "hormone" agar (Huntoon) with a primary reaction of P_H 6.3.

No better growth was obtained by the use of a medium containing a high concentration of amino acids than when prepared with the specified amount of peptone.

The presence of glucose does not enhance the growth of the gonococcus.

The growth stimulating principle in a medium prepared according to Huntoon's method was found to be slightly impaired by exposure in the autoclave to 120 C. for 5 minutes and seriously injured, but not entirely destroyed, after 30 minutes at that temperature.

Abundant moisture in the air of the incubator is a prime requisite for the optimal growth of the gonococcus, especially on first isolation, but a reduced oxygen tension was not found to be advantageous.

Fermentation tests constitute the most valuable single criterion for the differentiation of gonococci from other similar gram-negative diplococci. No one of 86 gonococcus strains tested split maltose, and all but one fermented glucose. None of the strains tested on levulose and galactose split these sugars. A sugarfree, semisolid, ascitic agar medium, with bromthymol-blue as an indicator, has proved satisfactory as a base for fermentation tests. Differential readings may be made after 18 to 24 hours' incubation.

A semisolid, sterilizable medium with a growth accessory factor (Huntoon formula) was found admirably adapted for carrying a large collection of gonococcus strains. Replantings have not been necessary oftener than once in three or four weeks.

Two plating mediums are described which have been found serviceable in the isolation of the gonococcus. One of these mediums is made in some degree selective by the incorporation of a dye, iodine-green. For the best results the reaction is of prime importance; the final H-ion concentration should be about 7.2. A method of collection of gonorrheal pus specimens for culture is described.

COMPARATIVE VALUE, FROM STANDPOINT OF
PUBLIC HEALTH, OF SMEARS, CULTURES AND
COMPLEMENT FIXATION IN THE DIAG-
NOSIS OF CHRONIC GONORRHEA
IN WOMEN *

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In the first part of this article are reported the results which have been obtained in the application of the cultural methods of Torrey and Buckell¹ to the diagnosis of cases of chronic gonorrhea in women, and in the second part, a comparative study of the relative values, from public health standpoint, of smears, cultures and complement-fixation tests in the diagnosis and control of such cases is presented. The patients were prostitutes who had received a court sentence.* *

1. *Cultural Results.*—As it was not feasible to seed the culture plates immediately after obtaining specimens from these cases, it was necessary to devise a procedure through which the viability of any gonococci present in the discharges, on the swabs, would be preserved during the period occupied in transporting them to the laboratory. A method which proved satisfactory is described in the article referred to. The plates should be poured at least 2 or 3 hours before they are to be streaked. After seeding, they are placed in an incubator in which the air is kept very moist and the temperature at 36 to 37 C. It is seldom worth while to attempt fishings from the plates after only 24 hours' incubation as the gonococcus colonies are frequently very small—barely visible to the naked eye—and not particularly characteristic. After 48 hours, however, the colonies have generally assumed a typical appear-

Received for publication April 24, 1922.

* This investigation was aided by a grant from the U. S. Interdepartmental Social Hygiene Board to New York University Medical College.

** The material for culturing was obtained either at the clinic connected with the Court or at the Kingston Avenue Hospital for Communicable Diseases in Brooklyn. The complement fixation tests were made by Miss M. A. Wilson, in charge of the Health Department Serologic Laboratory, and smear diagnoses by Dr. Catherine Regan, Bacteriologist at the Kingston Avenue Hospital. In correlating the work we have received assistance from Dr. W. H. Park and Dr. Anna Williams.

¹ *Jour. Infect. Dis.*, 1922, 31, p. 125.

ance and those resembling the gonococcus type should be fished. It is best not to delay the fishing from medium B plates beyond the second day as the gonococci tend to die out rather quickly on this medium. In some instances the gonococcus colonies have grown up very slowly, and accordingly all the plates should be incubated and inspected daily for at least 5 days. The isolation of the gonococcus in pure culture in some cases was a matter of considerable difficulty owing to the presence of large numbers of other colonies on the plates. Pure strains were always obtained, however, by replatings on one or the other of the dye-containing mediums. The appearance of the gonococcus colonies on these mediums, the method of fishing the colonies and the criteria for the identification of the gonococcus have been discussed in the preceding paper.

For each case 4 plates were generally used, prepared as follows:

1. Medium B, 10 c c plus 5 c c ascitic fluid plus 1 c c methyl violet, 1:100,000 dilution in distilled water.
2. Medium B, 10 c c plus 5 c c ascitic fluid.
3. Medium A, 10 c c plus 5 c c ascitic fluid plus 0.5 c c iodine-green, 1:3,000 dilution in distilled water.
4. Medium A, 10 c c plus ascitic fluid, 5 c c.

As is indicated in table 1, rather more successful isolations were effected with plates 3 than with any of the other combinations, although plates 2 yielded nearly as good results and in 4 instances showed gonococcus colonies when the other plates were negative. These 2 plates, however, were more frequently overgrown with contaminating bacteria.² In only 2 instances was a successful isolation obtained from plate 1 when the other 3 proved negative, and in many instances no gonococcus colonies developed on plate 1 when one or more of the other plates were positive. Streptococci gave a good deal of trouble and were present in much greater numbers in the specimens from the cervix uteri than in those from vulvovaginitis in children or from cases of urethritis in males. The methyl violet, in the strength employed, frequently did not inhibit the development of the streptococci nor certain prevalent types of diphtheroids, but too often did inhibit the growth of gonococci. It would seem, perhaps, hardly worth while to employ plates 1 in routine work on women. Plates 4, also, proved less effective than did the same medium with the dye, iodine-green. In view of these

² An effective method for limiting the overgrowth of plates with spreading types of bacteria (*B. subtilis*, etc.) consisted in ringing such colonies with gentian violet stain, applying the stain with a swab around the colony.

results we would recommend the use of mediums 2 and 3 in connection with gonorrheal cases of this type, employing at least one plate of the former and two plates of the latter for each specimen.

Of the total 102 women from whom cultures were made, successful isolations were effected in 29, or 28.4%.

TABLE 1
RESULTS OBTAINED WITH THE FOUR VARIETIES OF MEDIUMS EMPLOYED IN THE
ISOLATION OF GONOCOCCUS

Case	Plate 1 Ascitic Agar B + Methyl Violet	Plate 2 Ascitic Agar B	Plate 3 Ascitic Agar A + Iodin-Green	Plate 4 Ascitic Agar A
L. Sm.	—	+	—	—
L. S.	—	+	—	—
P. A.	—	—	—	+
E. B.	—	—	+	—
N. C.	—	—	+	+
M. D.	—	+	+	—
L. R.	—	+	+	+
L. D. cerv.	+	+	+	—
ureth.	+	+	—	—
S. B. cerv.	—	—	+	—
ureth.	+	—	—	—
A. R.	—	C	+	C
A. M.	—	+	+	+
G. S.	—	—	+	+
A. Mac.	+	+	+	+
F. H.	+	+	+	+
C. J.	—	—	+	—
J. F.	—	C	+	—
M. Do.	+	+	+	+
H. M.	—	+	+	—
R. G.	—	C	+	C
R. C.	—	—	—	—
A. D.	—	+	+	—
M. H.	—	—	+	—
M. W.	+	+	+	—
J. V.
V. M.	—	+	—	—
R. Ca.	C	C	C+	C
G. W.	—	+	—	—
V. G.	—	+	+	—
L. S.	—	—	+	—

+ Indicates gonococcus colonies on the plate; C indicates plate badly contaminated.

The strains isolated from our 29 positive cases were definitely identified as gonococci. In 5 instances pure cultures were obtained and identified on the basis of type of colony growth, morphology, and reaction to gram stain, rapid autolysis and short period of viability, but the strains were lost before further tests could be applied. The other 24 strains were all subjected to fermentation tests and a considerable number of them to agglutination tests with a polyvalent gonococcic serum. We believe there can be no question in regard to the identity of them all.

From the standpoint of control of venereal infection it is of interest to find that 9 of the 29 patients with positive cases harbored the gonococcus in the cervix uteri without any definite symptoms of gonorrhea. Also, although in most of these positive cases the number of viable gonococci was very small, there was no definite correlation between the number of gonococcus colonies on the plates and the clinical picture; that is to say, a few of the clinically doubtful cases gave fairly numerous gonococcus colonies on the plates, whereas certain other cases showing quite definite clinical symptoms yielded exceedingly few gonococcus colonies and those only on one plate. We have made no comparative virulence tests on the gonococcus strains isolated. It is possible that those from the clinically doubtful cases would prove to be relatively avirulent. Jøtten³ has recently reported that strains from cases of gonorrhea with complications are, as a rule, much more virulent, as indicated by the inoculation of white mice, than those cases with milder symptoms.

In regard to 92 of the patients there are official records of the results of gram-stained smears. Accordingly, we may present at this point a comparative statement bearing on the relative values of these two methods of diagnosis. In most instances smears from the urethra and the cervix uteri were prepared at the time the specimen for culture was obtained. In addition, from most of the cases smears were taken at other times. In the tabulation the report most suggestive of gonorrhoeal infection has been selected. In diagnosing these smears the rules of Williams and Wilson⁴ were followed: positive smears show leukocytes filled with morphologically typical gonococci; suspicious smears show some intracellular organisms suspiciously like gonococci and 50% or more of polymorphonuclear leukocytes; observation smears show 50% or more of polymorphonuclear leukocytes, but no suspicious intracellular diplococci.

Of these 92 different cases, 26, or approximately 28%, yielded positive cultures. Among these cases there was one acute infection and 8 subacute. The majority, or 59, presented the clinical picture of chronic gonorrhea, as described by Smith and Wilson,⁵ with at least 21 cases for which a diagnosis of "doubtful gonorrhea" was given. Gram-stained smears from these patients yielded a positive diagnosis in 13

³ München. med. Wchnschr., 1920, 67, p. 1067.

⁴ Collected Studies from Research Laboratory, Dept. of Health, City of New York, 1911, 6, p. 29.

⁵ Jour. Immunol., 1920, 5, p. 499.

instances, or in just one-half as many as did the cultural method. Of the 26 culturally positive cases, 5 gave positive films, 2 were suspicious, and 19 were reported as of the observation type. There were, accordingly, 8 cases yielding positive smears from whom the gonococcus was not isolated.

Twenty-six of the patients had received irrigations for longer or shorter periods before the cultures were made, although, of course, the local treatments were stopped 3 or 4 days before the specimens were taken. Of these treated patients only 3 yielded positive cultures, but 5 gave positive smears. Further, the 5 cases giving positive smears were not among those successfully cultured; of the latter, 2 gave the observation type of smear and 1 the suspicious type.

It should be noted that in some of these cases the plates were so overgrown with other types of bacteria that gonococci, even if present, would not have had an opportunity to develop. If a second examination had been made in some of these cases no doubt a somewhat higher percentage of positive results might have been obtained. On the other hand, the smears were not always of such a quality as to provide optimal conditions for examination.

These results are summarized in table 2.

TABLE 2
COMPARATIVE RESULTS WITH CULTURAL AND SMEAR METHODS OF EXAMINATION

Total Number of Cases	Cultural Results		Gram-Stained Smears			
	Positive	Negative	Positive	Suspicious	Observation Cases	Negative
92	26	66	13	17	56	6

From our results with these two methods of diagnosis in suspected gonorrheal infections of this type we may conclude that the cultural methods employed are likely to give a higher percentage of definitely positive findings than are the smear examinations, but on the other hand, the latter may be positive when cultures are negative. Of the 21 cases clinically diagnosed as "doubtful gonorrhea," 5 gave positive cultures and 3 positive smears and 4 suspicious smears, but 4 out of the 5 culturally positive cases yielded the observation type or negative smears. These two methods, thus, tend to supplement each other and are both of value in the control of these cases.

2. *Comparison of the Complement-Fixation Test with Smear and Culture in Relation to Clinical Diagnosis.*—In this section are reported the results obtained with complement-fixation tests by Miss Wilson of the Serologic Laboratory, Department of Health, the smear examinations at the Kingston Avenue Hospital, and the cultures by Mr. Buckell, on 56 of the 102 cases described in part 1.

The method for the complement-fixation test has been described.⁵ The essential points are the preliminary tests of individual guinea-pig serums for gonococcus fixability and careful titration of the selected, pooled complement with constant doses of antishoop amboceptor and 5% suspension of sheep cells.

TABLE 3

COMPARISON OF SMEAR, CULTURE AND COMPLEMENT FIXATION IN VARIOUS STAGES OF GONORRHEA IN WOMEN

Diagnosis	Number of Cases	Percentage of Positive Diagnoses		
		Smears	Cultures	Complement Fixation
Acute gonorrhea.....	1	Observation	100% positive	No complete fixation
Subacute gonorrhea.....	8	50%	50%	50%
Chronic gonorrhea.....	33	12%	20%	69.5%
Doubtful gonorrhea.....	14	14%	28%	71%

A summary of the tests of the 56 cases without reference to the clinical data, shows 16 cases giving positive cultures, or 27%, 10 giving positive smears, or 16.5%, and 37 giving fixation, or 66%. The fixations were divided into: 8 strongly positive, or 14%; 15 moderately positive, or 26.5%, and 14 weakly positive, or 25%. If we should consider the weakly positive reactions insignificant, we would still have 40.5% positive fixations in the 56 cases. In the discussion of the following tables we shall give our reasons for considering the weakly positive reactions to be diagnostic of present or recent gonorrheal infection.

The important point is to determine the relation of the laboratory tests to the clinical classification, and we give a comparison in table 3. The clinical classification is that used at the Kingston Avenue Hospital as described by Smith and Wilson.⁵ This classification cannot be taken as a hard and fast guide to the course of the disease because of the inability to obtain correct histories and dates of infection of the patients.

As was demonstrated by Schwartz and McNeil,⁶ in the acute and subacute stages of gonorrhea one would not expect to obtain as high a percentage of positive reactions as in the chronic stage.

It is of interest to compare our results with those of Smith and Wilson, who carried out a similar comparative investigation, using for the cultural work a glycerol-veal-horse-serum agar streaked with blood. Of a total of 50 cases, 7 were positive culturally, 14%; 3 were positive by smear and 26 had smears of the observation type; 41 gave positive fixation, or 82%, with 8, or 16%, showing a strong positive reaction.

Wilson has made a further study (to be published) of 181 cases previous to the present series. The 181 cases were classified as:

54 subacute cases⁷ having 46.5% positive smears,
49.5% positive cultures,
51.5% positive fixation.

85 chronic cases having 13% positive smears,
15% positive cultures,
76% positive fixation.

7 doubtful clinically no positive smears,
no positive cultures,
42% positive fixation.

Controls: 346 nongonorrheal patients gave no trace of fixation, and 4 normal laboratory workers gave no trace of fixation.

In these three studies the complement fixation reactions in chronic cases dropped from 82% in first study to 76% in second study and to 69.5% in the present study.

We have not been able to determine the exact reason for this decrease in the positive reactions, but the chief cause may have been due to the time in the course of the disease that the tests were made. The character of the patients in our service has changed somewhat during the past two years. At the time of our first study all prostitutes having venereal disease were sent to Kingston Avenue Hospital, while, for some time past, the first offenders and young girls, only, are sent there.

In table 4 is given a report of the laboratory tests on 16 of our culturally positive cases. In these the complement-fixation tests were made at the serologic laboratory. The remainder of our culturally positive cases had the complement-fixation tests performed at another laboratory and are not included in this table.

⁶ Ann. Jour. Med. Sc., 1912, 144, p. 815.

⁷ Included in the 54 subacute cases are 11 diagnosed as acute on admission, but as these were not early acute they have been placed with the subacute cases.

TABLE 4

RESULTS OF COMPLEMENT FIXATION AND SMEAR EXAMINATIONS IN 16 CASES
CULTURALLY POSITIVE

Case	Complement Fixation			Smears	
	Positive	Negative	Doubtful	Urethra	Cervix
L. S.	+	Suspicious	Suspicious
L. Sen.	+	+	+
P. A.	3+	Observation	Observation
E. B.	—	..	Observation	Observation
N. C.	3+	Observation	Observation
M. D.	+	Suspicious	Suspicious
L. R.	—	..	Observation	—
L. D.	3+	Observation	Observation
A. R.	—	..	Observation	Observation
A. M.	+	Observation	Observation
A. Mac.	+	Observation	Observation
J. F.	±	Observation	Observation
J. V.	2+	Observation
V. M.	3+	—	Observation
R. Ca.	—	..	Observation	Observation
M. H.	2+	Observation	Observation

In table 5 is given a comparison of the laboratory tests in 43 culturally negative cases.

TABLE 5

RESULTS OF COMPLEMENT FIXATION AND SMEAR EXAMINATIONS IN 41 CULTURALLY
NEGATIVE CASES

Cases	Complement Fixation*			Smears†		Smear at Time of Culture	Culture Material
	Positive	Negative	Doubtful	Urethra	Cervix		
M. K.	—	..	Observation	Observation	Observation	Cervix
H. B.	—	..	+	—	—	Cervix
M. Kar.	±	+	—	—	Cervix
M. W.	—	..	Suspicious	Observation	—	Cervix
G. B.	2+	Suspicious	Observation	Observation	Cervix
A. H.	2+	Suspicious	Observation	Observation	Cervix
B. M.	2+	+	—	—	Cervix
E. W.	+	Observation	—	—	Cervix
M. A.	—	..	+	—	—	Urethra
M. S.	4+	Observation	—	Observation	Cervix
R. G.	+	Observation	—	—	Cervix
T. F.	2+	Observation	—	—	Cervix
J. O'C.	—	..	Observation	—	—	Cervix
V. H.	3+	Observation	—	Observation	Cervix
H. Ba.	2+	Suspicious	—	Observation	Urethra
W. B.	—	..	Observation	—	—	Cervix
A. B.	—	..	Suspicious	Observation	—	Cervix
M. B.	+	Observation	—	—	Cervix
C. C.	3+	Observation	Observation	Observation	Cervix
M. C.	+	Observation	Observation	Observation	Cervix
M. D.	2+	+	Observation	—	Cervix
B. E.	2+	Observation	Observation	—	Cervix
M. G.	—	..	Observation	—	—	Cervix
H. A.	—	..	Suspicious	—	—	Cervix
V. J.	+	+	Suspicious	—	Cervix
L. L.	3+	Observation	Suspicious	Suspicious	Gland
D. M.	+	Suspicious	Suspicious	—	Cervix
H. P.	+	Observation	Observation	—	Urethra
F. F.	—	..	Observation	Observation	—?	Cervix
I. F.	2+	+	+	Observation	Cervix
M. A.	2+	Observation	Observation	—	Cervix
A. E.	±	Observation	Observation	—	Gland
V. R.	+	+	Observation	—	?
J. R.	—	..	—	—	—	Cervix
E. C.	2+	Observation	Observation	Observation	Cervix
H. M.	2+	Suspicious	Suspicious	?	?
M. M.	—	..	Observation	Observation	Observation	Cervix
E. W.	+	Observation	Observation	—	Cervix
E. LaR.	2+	Observation	Observation	—	Urethra
S. G.	—	..	Observation	—	Observation	Cervix
B. F.	2+	—	—	—	Urethra

* Result of fixation at height of reaction is given. From 3 to 23 tests were made on each case, with an average number of about 8.

DISCUSSION

The results of this investigation tend to confirm the general impression that neither clinical observations alone, cultural tests, gram-stained smears nor fixation tests, as single methods of diagnosis, can be relied on as guides to diagnoses of actual infection with the gonococcus in cases of suspected chronic gonorrhea in women.

Considering first clinical observations, we find that 9 of our 29 culturally positive cases harbored the gonococcus in the cervix uteri without exhibition on the part of the patients of symptoms definite enough to warrant a clinical diagnosis of gonorrhea. Also 7 other cases, diagnosed as doubtful gonorrhea, which were culturally negative, gave definitely positive complement fixation and two of them also positive smears. Conversely, among 33 cases given a diagnosis of chronic gonorrhea, there were 5 cases in which the clinical diagnosis was supported neither by the cultural test nor by repeated fixations and smears.

Diagnosis by cultural tests would be, of course, the preferred laboratory procedure if it might be carried out with the facility and confidence which that method enjoys as applied, for instance, to the diagnosis of diphtheria. This, however, is at present quite far from being the case. Although our cultural procedures, as applied to the diagnosis of chronic gonorrhea in women, have yielded results which seem perhaps to be better than any reported heretofore, among 41 culturally negative cases (table 5), which were carefully studied, there were 8 cases giving positive smears and 13 other patients who gave a 2+ to 4+ complement fixation. In justice to the cultural method, however, it should be noted that in 6 of these 8 smear-positive cases, the smears from the urethra alone were positive, whereas the material for culture was obtained from the cervix uteri; also that the results given in table 5 for fixation and smear examinations were the optimal ones of repeated tests, whereas in the great majority of these patients only a single cultural test was made.

As regards diagnosis by means of gram-stained smear examinations our results would seem to indicate that this method would be likely to yield a considerably smaller percentage of definitely positive diagnoses than the cultural methods which we have employed. The relation of the findings with these two methods is summarized on page 151. It is stated there that 19 of the 26 culturally positive cases gave only the observation type of smear and, on the other hand, that many of the culturally negative cases also gave this type of smear. These observation smears,

however, give no more definite information than their name implies, namely, merely denoting the presence of an inflammatory condition in the urethra or cervix uteri, due to some pyogenic organism, quite possibly to the gonococcus. It is not unlikely, in fact, that secondary infections of these localities by streptococci of low grade virulence or other similar organisms persisting after the disappearance of the gonococcus might well be accountable for some of these observation types of smears and hence, if much dependence is placed on this finding, the release of such cases might be delayed longer than is necessary.

As regards the fixation test, there has been included in our list of positive gonococcus diagnoses all of the one-plus or weakly positive reactions. This has been done because in Miss Wilson's two previous studies^{5,7} it has been demonstrated that this test is highly specific and that even weakly positive reactions may be considered as diagnostic of a gonorrheal infection. In these studies it is reported:

1. No degree of complement fixation was obtained in cases known as nongonorrheal. Those controls included 345 cases diagnosed as nongonorrheal and 4 normal laboratory workers.

2. The reaction reported as one-plus represents a definitely positive result and has had, in some instances, a comparative reading by a representative of another laboratory of 2-plus or even higher.

3. Some of our one-plus reactions have persisted for from 5 to 11 weeks. These tests were carefully controlled and could not be considered as "doubtful" from the point of view of technic.

4. The clinicians of the Venereal Disease Service of the New York City Health Department, as the result of a long series of observations, have come to regard a one-plus reaction as diagnostic of gonococcus infection. To quote Dr. Barringer's opinion in a recent paper:⁸ "Further study of groups of cases with special reference to undetected foci of gonococcus infection may establish the fact that the complement fixation test is the surest means of estimating when a cure has been effected and that we are justified in keeping the patient under treatment as long as these tests remain positive."

It would seem, then, that in the chronic stage of gonorrheal infection and also in the clinically doubtful cases complement-fixation tests, carefully controlled, will give a much higher percentage of positive diagnoses than cultures or smears (table 3) and that this test constitutes at present the simplest and most effective single guide for the control of

⁸ N. Y. State Med. Jour., 1922, 22, p. 145.

such cases. On the other hand, it should be noted that some positive cases will be missed if dependence is placed on complement fixation and smear examinations alone. As is shown in table 4, four of the 16 culturally positive cases (25%) gave persistently negative fixation tests and also indefinite smears (observation cases). The clinical diagnosis for 3 of these cases was chronic gonorrhea and for the other "doubtful gonorrhea." Then, too, a persistent one-plus reaction may mean in some cases past rather than present infection with the gonococcus. We do not know how long the antibodies may linger in the body after the period of actual infection has terminated.

SUMMARY

By way of a general conclusion it may be stated that the smear, cultural and complement-fixation methods of diagnosis in chronic gonorrhea in women have all proved useful, and that their relative values correspond to the order in which they are named, the last being the most valuable. Whenever possible, however, each test should be carried out, as it is shown that they tend to supplement each other.

It would seem likely that the cultural methods utilized in this study might find their most useful application, as far as public health work is concerned, in controlling the period of detention of infected women undergoing treatment and also in determining when cases of vulvovaginitis in children may be pronounced cured. With all such patients, of course, local treatments should be stopped at least 4 or 5 days before the cultures are made. In women patients material should be obtained for culture from both the urethra and the cervix uteri. At best it must be admitted that the conditions essential for the isolation of the gonococcus from these chronic infections of women are exacting and can be met only by one experienced in bacteriologic technic and with the facilities of a well-equipped laboratory. In spite of the greatest precautions, too, the plates at times may become overgrown with contaminating bacteria. The method is also more time-consuming than are the smear and complement-fixation procedures. A positive report cannot be made, at the earliest, before 2 days and for a negative report from 4 to 5 days may be required. On the other hand, the isolation of the gonococcus from one of these patients answers the question of infection in an entirely definite way and, under certain conditions, the results obtained may well repay the time and trouble necessary for the application of these cultural procedures.

THE PATHOGENICITY OF *B. MELITENSIS* AND *B. ABORTUS* FOR GUINEA-PIGS

STUDIES ON THE GENUS *BRUCELLA* NOV. GEN. IV

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In a preliminary communication¹ it was demonstrated that 5 old stock strains of *B. melitensis* inoculated by the intratesticular route produced in guinea-pigs anatomic lesions, which could not be distinguished from those seen in this species infected with *B. abortus*. In fact, the pathogenicity tests furnished additional evidence to support the contention of recent workers that *B. melitensis* and *B. abortus* are closely related. It was shown that the etiologic agent of undulant fever of man persists for at least 2 months in the tissues of infected animals and exhibits a peculiar affinity for the spleen, lymphnodes, liver and sex organs. Comparative serologic studies with the serums of the infected animals and the cultures obtained from their organs conclusively disproved any assumption that the infections might have been caused by accidentally ingested abortion bacilli, instead of by the inoculated *B. melitensis*. In order to verify the preliminary observations, a total of 22 strains of *B. melitensis*, obtained from English, Algerian and Italian laboratories, was subsequently tested on guinea-pigs. Some of the findings necessitated a comparative study of the lesions produced by *B. abortus*. It has therefore been considered advisable to include in this paper data on the experimental guinea-pig disease produced by the bacteria of the *Brucella* group, which have been collected in this laboratory during the last 6 years.

METHODS

The cultures used to inoculate the guinea-pigs were grown on glycerol peptic digest agar (P_H 6.8-7.2); only the tubes seeded with *B. abortus* cultures were sealed. The injections were made either into the abdominal cavity or into the parenchyma of the testicle. The latter mode of infection was carried out as follows: The anterior limbs and the left leg of the guinea-pig were held by an assistant,

Received for publication, April 26, 1922.

¹ Proc. Soc. Exper. Biol. and Med., 1919, 16, p. 757.

and the animal was placed on its back. The operator fixed with the left hand the right or left testicle in the scrotum; occasionally gentle pressure on the abdominal wall close to the pelvic ring was necessary to bring the organ into proper position. The skin was thoroughly cleansed with alcohol and ether and then painted with iodine, or better, with a mixture of brilliant green and crystal violet in 50% alcohol; a fine needle was inserted through the scrotum into the parenchyma and, as a rule, 0.2-0.5 cc of the bacterial suspension was injected. The fingers of the left hand controlled the location of the needle and the intra-testicular tension; particular care was exercised to prevent a rupture of the punctured testicle by the rapid injection of excessive amounts of the inoculum. The needle puncture was closed with iodized collodium.

The course of the infection was followed by regular weight determinations and skin tests. The preparation of the antigens, the method of application and the interpretation of the cutaneous reactions have been stated in the previous papers.² When the animals were considered ready for bacteriologic examinations, they were chloroformed, necropsied and the lesions noted; portions of the tissues were preserved for sections, and cultures were prepared from the spleen, liver, kidneys, lymphnodes, bonemarrow, urine, bile, abscesses and bone lesions. Sheep-blood-peptic digest agar plates and peptic digest agar slanted were the mediums employed. Pieces of tissue of guinea-pigs infected with *B. abortus* were rubbed over the entire surface of the agar slant, while sections of organs of the animals inoculated with *B. melitensis* were spread over blood plates. The tubes were sealed with paraffin. The colonies were identified by microscopic examination and by carefully conducted agglutination tests. Specific anti-serums unabsorbed and absorbed with different strains were continuously used, and the procedures were followed, as outlined in paper II.³ Complete agglutination tests were also carried out with the serum of the sacrificed guinea-pigs. It is evident that the existence of an infection in the guinea-pigs was considered proved only by the presence of (1) positive skin reactions; (2) distinct anatomic changes in the spleen, the liver, the lymphnodes, the testes, etc.; (3) microscopic changes in the form of focal lesions consisting of nests of actively growing epithelioid cells and connective tissue hyperplasia; and (4) positive serum reactions, particularly agglutination reactions in dilution

² Amer. Jour. Dis. Child., 1918, 36, p. 268; 1919, 38, p. 577.

³ Feusier and Meyer: Jour. Infect. Dis., 1920, 27, p. 185.

above 1:100.⁴ The nature of the etiologic agent responsible for the above lesions and reactions, whether *B. abortus* or *B. melitensis*, was decided by (5) isolation of the bacteria from the tissues; (6) identification of the organisms by agglutination tests with serums specific for the various groups of the genus *Brucella*; (7) by absorbing the serum procured from the infected guinea-pigs with various antigens; and (8) by "passage" experiments. In the latter tests, either cultures obtained from the tissues on the first generation or the emulsions of portions of the organs were inoculated into guinea-pigs. Previous tests have demonstrated that *B. abortus* infections are always successfully perpetuated in this manner and that, as a rule, the anatomic lesions become more striking in the course of these "passages."

Early in the course of the experiments, the observations of Surface⁵ on spontaneous *B. abortus* infections among guinea-pigs under crowded laboratory conditions were recalled and caused a feeling of uncertainty. In spite of the fact that such spontaneous epizootics, due to organisms of the *Brucella* group, had not been encountered among the many thousands of guinea-pigs observed during the last six years in our laboratory, the laborious and time-consuming application of all the methods mentioned was considered necessary in order to avoid even the suspicion of possible spontaneous infection. Eliminating all conceivable sources of error, the data which will be presented conclusively demonstrate that *B. melitensis* can provoke in a certain percentage of guinea-pigs an infection indistinguishable from abortion disease.

EXPERIMENTS WITH *B. MELITENSIS*

Most of the publications on undulant fever convey the impression that attempts to excite a lethal infection in the usual laboratory rodents are for the most part unsuccessful, and in order to produce a fatal disease it is necessary to introduce enormous numbers of organisms subcutaneously or intraperitoneally. It is said that the infection follows a protracted course, and weeks, even months, may pass before death occurs. A careful review shows that at least for the guinea-pig certain definite lesions have been noted by a number of observers.

Durham,⁶ in 1898, by intraperitoneal injections, produced in guinea-pigs a number of infections which were chronic in character. He demonstrated *B. melitensis* in the urine and in the intensely engorged kidneys of these animals, which died or were killed long periods (74-94 days-7 months) after inoculation.

⁴ Repeated cutaneous injections of bacterial proteins may cause the appearance of agglutinins.

⁵ Jour. Infect. Dis., 1912, 11, p. 464.

⁶ Jour. Pathol. & Bacteriol., 1899, 5, p. 377.

In two instances, the spleen was much enlarged, but in the others the organ showed nothing abnormal; however, the animals were invariably emaciated. The serum of the inoculated guinea-pigs agglutinated *B. melitensis* in high dilutions (for example, 51st day, 1:200; 74th day, 1:1000). The same worker also injected guinea-pigs by the subdural route with cultures whose virulence had been increased by previous intracerebral passage, a method which had originally been used by Cantani to raise the virulence of *B. influenzae*. The course of the infection was acute, and in the animals that died the organisms were isolated from the viscera.

In 1904, Carbone,⁷ who later died of undulant fever contracted in his laboratory, showed that male guinea-pigs often developed a purulent inflammation of the tunica vaginalis, following intraperitoneal injection of *B. melitensis*.

It is to Eyre,⁸ however, that most credit is due for having studied the *B. melitensis* in relation to guinea-pig infections. He showed that following the first isolation from human cases, the *B. melitensis* could kill guinea-pigs only when given subcutaneously, intravenously or intraperitoneally in tremendous doses. Infection was also possible by intracranial inoculation. Using a freshly isolated strain, the infection was chronic, the animals living from 100 to 200 days. By repeated intracranial injections, he was able to increase the virulence of the organisms, so that the pigs died within 24 to 72 hours after an intravenous injection, 3 to 12 days after an intraperitoneal inoculation and 9 to 36 days after an intracranial inoculation.

Eyre described two types of infection in guinea-pigs, the acute and chronic, as follows:

Acute Melitensis Infection.—"Several days after a moderate dose of a highly virulent culture, or a heavy dose of a less virulent culture, the animal dies. For from two to three hours after the injection, he seems to be all right and eats well, but loss of weight sets in, which is highly characteristic for the disease. At first, the temperature drops rapidly, then rises. The animal sits huddled up, will not eat, becomes weaker and, if disturbed, goes into tonic convulsions. This stage of increased irritability goes gradually into a stage of coma, from which the animal can be aroused with difficulty, and in which he remains about 24 hours with a subnormal temperature. Sometimes he has a convulsion before death, but usually it occurs without warning. The postmortem findings are those of acute septicemia. The organisms can be recovered from all the tissues and organs. In male animals, following intraperitoneal infections, there develops a bilateral purulent process in the tunica vaginalis with beginning hypertrophy of the testes. This pus contains numerous organisms."

Chronic Melitensis Infection.—"With small doses of virulent, or fair doses of moderately virulent organisms, the animal gets a chronic infection. Aside from a progressive loss of weight and a severe anemia, there are no characteristic symptoms. Even the temperature is not much changed. The incubation period of the chronic variety of the disease is from two to three days. Then comes a time, three to six days, when the animal is perceptibly sick. He will not eat, stays in a corner of his cage, loses weight and is very weak. He gradually improves, eats well, even ravenously, and although losing no more weight, he never regains his original weight. Following weeks or months, during which, aside from the loss of weight, the animal seems well, he suddenly dies. Post-mortem cultures from the liver are sterile; from the spleen and bonemarrow there is sometimes a scanty growth, although these are also often sterile. From the kidneys and the urine a good growth of *B. melitensis* is obtained."

⁷ Arch. per le sc. med., 1904, 28, p. 273.

⁸ Report Mediterranean Fever Commission, 1905, Part 2, p. 67.

Nicolle and Conseil,⁹ in 1909, reported on 2 guinea-pigs, which had naturally acquired a *B. melitensis* infection in a stable, where infected maltese goats had been kept. The serum of both animals agglutinated *B. melitensis* in a dilution of 1:300 and the specific organism was isolated from the spleen and liver of one guinea-pig. The blood, urine and bile were sterile. In a subsequent paper¹⁰ these two investigators recorded some inoculation experiments on guinea-pigs with a strain recently isolated from the milk of an infected goat and they stated that "Contrairement à l'opinion classique, nos expériences ont donné constatement un résultat positif; le cobaye est donc un animal parfaitement sensible au *M. melitensis*." One guinea-pig, which had been injected with the growth of one ¼ agar slant and sacrificed on the 138th day, exhibited a slight hypertrophy and a granular appearance of the spleen. The same findings were made on a similarly treated guinea-pig, which was killed on the 42nd day. Feeding of cultures to 2 guinea-pigs for 10 consecutive days produced the same lesions. Spleen cultures were always positive, while *B. melitensis* was also isolated from the urine of 2 animals. The serum agglutinated the inoculated organism in dilutions of from 1:250 to 1:1000.

Sangiorgi (1913)¹¹ inoculated 3 guinea-pigs intraperitoneally with the blood of an undulant fever patient. The blood of 2 of the animals, when killed on the 30th day, agglutinated *B. melitensis* in a dilution of 1:100, and the spleens were greatly enlarged and soft ("la milza era molto in grandita, friabile"). In a recent study by Tallo,¹² it is shown that *B. melitensis* may persist for at least 35 days in the gallbladder and bile of guinea-pigs, which have been injected intravenously. These observations confirm those of Eyre and others and show that *B. melitensis* may leave the body by way of the alimentary canal, in the intestinal mucus and in the feces.

It is evident, from this review, that guinea-pigs are susceptible to *B. melitensis* and that a chronic disease with an enlargement of the spleen and a persistence of the inoculated bacteria in the tissues and secretions has been repeatedly noted. The significance of these facts has, however, not been appreciated until a study of *B. melitensis* by guinea-pig inoculations. Preliminary experiments,¹ conducted by the writers, resulted in negative postmortem and cultural findings until a special mode of injection, namely, the intratesticular route, was chosen. Some observations, which will be detailed in a subsequent chapter, had indicated that *B. abortus* possessed a striking affinity for the sex organs. As the two bacteria, *B. abortus* and *B. melitensis*, were morphologically, biochemically and serologically closely related, it was suspected that the sexotropic pathogenicity of *B. abortus* might also be characteristic of the organism of undulant fever and the possibility, therefore, of producing specific lesions by injections of the virus into the testes suggested itself. The majority of the inoculations reported in the following were

⁹ Compt. rend. Soc. de biol., 1909, 66, p. 503.

¹⁰ Ibid., 1909, 67, p. 267.

¹¹ Pathologica, 1913, 5, p. 554.

¹² Ibid., 1919, 11, p. 401.

made intratesticularly. Successful infections have, however, also been noted following intraperitoneal injections. The data dealing with the observations on 203 guinea-pigs are also presented in the following.

EXPERIMENTS WITH *B. MELITENSIS* STRAINS 1, 2, 3, 4, 5, 7,
8, 9, 10, 23, 24 and 25¹³

One tenth slant of a glycerol peptic digest agar culture was inoculated into the right testicle of guinea-pigs; as a rule, a series of 4 to 6 animals were infected. A total of 60 guinea-pigs was used; they all survived the infection and were sacrificed at different times after the inoculation, the period varying from 6 weeks to 6 months. Repeated skin tests gave negative reactions. At necropsy, the tissues were free from lesions, and the serum failed to agglutinate the strain of *B. melitensis* used for the inoculations in dilutions greater than 1:40.

EXPERIMENTS WITH A POLYHOMOGENOUS MIXTURE OF *B. MELITENSIS*
STRAINS 2, 3, 4, 5, AND 11

The results on the first series of 8 guinea-pigs have already been reported in a previous communication.¹ The cultures obtained from the tissues of 2 guinea-pigs (614 and 615) which exhibited typical necropsy findings were used for further pathogenicity tests. The results are tabulated below.

MELITENSIS CULTURE FROM URINE AND TESTIS OF GUINEA-PIG 614
INOCULATED INTRATESTICULARLY

Guinea-Pig 654.—Sacrificed on 119th day; skin tests always positive; necropsy revealed small spleen, but typical lesions in lymphnodes and liver.

Guinea-Pig 655.—Died on 45th day with typical lesions.

Spleen emulsion inoculated with negative results intratesticularly into four (709, 710, 711, 712) and intraperitoneally into two guinea-pigs (713, 714).

Spleen culture, 2d generation, was identified in Monkey 15 as a *B. melitensis*. One guinea-pig (733), of 8 inoculated, revealed a typical lesion at necropsy.

The histories of guinea-pigs 655 and 733 are presented in detail.

Guinea-Pig 655.—Weighing 540 gm., was inoculated into the right testicle with 1/20 slant of a young culture (2d generation) of *B. melitensis* isolated from the testis and urine of guinea-pig 614. On the 44th day after the inoculation (weight 537 gm.) the animal was subjected to a skin test with "aborto" and "melitensis protein." The following day, the guinea-pig exhibited marked skin reactions, coryza and dyspnea; it apparently died from anaphylaxis 21 hours after the cutaneous injection of the bacterial proteins. Necropsy demonstrated

¹³ Histories of Strains: 1: marked R. A. M. C.; 2: U. S. Navy; 3: stock 22; 4: Dr. Arneth; 5: stock; all obtained from the Hygienic Laboratory, U. S. Public Health Service 1918; 7: marked R. Mum. 5; 8: marked R. Monaco; 9: R. B₂ paramelitensis, obtained from Dr. E. Sergent, Institut Pasteur d'Algerie, 1919; 10: obtained from the Institute for Infectious Diseases, Bern, 1914; 23: paramelitensis R. A. M. C., obtained from the Lister Institute; 24: marked Celli obtained from Dr. A. Ascoli, Modena, Italy; 25: marked "Douglas" obtained from Dr. J. W. H. Eyre, Guy's Hospital, London, 1919. *

an enlarged, nodular and typical spleen (weight 1.9 gm.), large and firm lymphnodes, numerous foci in the liver and bilateral atrophy of the testes. The microscopic findings were characteristic. *B. melitensis* was isolated from the spleen and the left precrucial lymphnodes; the urine, bile, bonemarrow, liver and testes were sterile. The serum gave the following agglutination reactions:

B. melitensis 1, 2, 3, 4, 5, 8, 11 — 1:400 = 1:1000 depending on the antigen; after absorption with *B. abortus* 80, 1:200.

B. melitensis 7 and 9 < 1:40.

B. abortus 80 and 320 — 1:100; after absorption with *B. abortus*, < 1:40.

Guinea-Pig 733.—Weighing 450 gm., was inoculated intratesticularly with one 1/10 slant of a culture (1st generation) obtained from the spleen of guinea-pig 655. The skin reactions were positive. On the 155th day, the animal weighed 221 gm. and was chloroformed. The necropsy findings were typical, the spleen weighed 1.4 gm., and the liver was studded with small, grayish foci. *B. melitensis* was isolated from the spleen and bonemarrow and the serum agglutinated *B. melitensis* 11, 1:1000, *B. abortus* 80, 1:800 and *B. melitensis* 7 and 9, < 1:20.

Two guinea-pigs (706 and 707) inoculated with the culture obtained from the urine of guinea-pig 615 survived and presented negative necropsy findings. A third guinea-pig (708) died on the 30th day after the inoculation with typical lesions and positive cultural findings.

A second series of 12 guinea-pigs gave the following result: Four of 6 guinea-pigs inoculated intraperitoneally with 2,000 million bacilli of a mixture of *B. melitensis* strains 2, 3, 4, 5 and 11 died on the 2nd and 3rd days, respectively, after the inoculation. In every case a fibrinopurulent peritonitis was present, and the injected bacilli were isolated from every organ. The remaining 2 guinea-pigs proved negative at necropsy on the 120th day. Six guinea-pigs injected intratesticularly with the same inoculum survived and presented negative findings when sacrificed 112 days after the inoculation.

A third series of 4 guinea-pigs inoculated intraperitoneally with 1/50 slant of the mixture mentioned of strains furnished 2 guinea-pigs (807 and 809) with moderately severe chronic lesions. Two guinea-pigs died on the 16th and 26th day, respectively; *B. melitensis* was found in the spleen, liver, bonemarrow, bile, kidneys and urine, but not in the heart blood. The histories of guinea-pigs 807 and 809 are detailed.

Guinea-Pig 807.—Weighing 330 gm., was inoculated intraperitoneally with 1/50 slant of a mixture of *B. melitensis* 2, 3, 4, 5 and 11. It was chloroformed 87 days after the inoculation when it weighed 417 gm. The spleen weighed 3.5 gm., it was nodular and soft; the edges were rounded. Numerous lymphnodes, in particular the iliac, precrucial and postmanubrial nodes, were slightly enlarged and firm. A few *B. melitensis* were isolated from the splenic pulp; the bonemarrow, liver, bile, urine, and kidneys were sterile. The serum agglutinated the various strains as follows: *B. melitensis* 11, 1:100-200; *B. melitensis* 7, 1:80; No. 9, 1:20; and *B. abortus* 80, 1:200; after absorption with *B. abortus* 80, the serum agglutinated *B. melitensis* 1:60. The strain of

B. melitensis isolated from the spleen was agglutinated in a dilution of 1:200 by a group II serum after the serum had been absorbed with *B. abortus* No. 80 (serum titer 1:1000, after absorption 1:200 for *B. melitensis* group II and *B. abortus* 80, <1:40). An emulsion of the spleen was inoculated intraperitoneally or intratesticularly into guinea-pigs 877, 878 and 879.

Guinea-Pig 809.—Weighing 685 gm., was inoculated intraperitoneally as guinea-pig 807. It gave a strongly positive skin reaction with *B. melitensis* and *B. abortus* protein on the 76th day after the injection. The guinea-pig was sacrificed on the 97th day when it weighed 676 gm. The postmortem findings were typical; the spleen weighed 2.2 gm.; it was nodular and pulpy. The lymphnodes were enlarged and hard. *B. melitensis* was isolated from the spleen, while the urine, bile, bonemarrow, etc., were sterile. The serum agglutinated the various *B. melitensis* strains as follows: *B. melitensis* 11, 1:200; *B. melitensis* 7 and 9, 1:100; and *B. abortus* 80, 1:200; when absorbed with *B. abortus* No. 80, the serum agglutinated only *B. melitensis* 11 in a dilution of 1:100.

In a fourth series the infected spleen of guinea-pig 807 was injected intraperitoneally into guinea-pigs 877 and 878, while guinea-pig 879 was treated intratesticularly. The three animals presented typical lesions when sacrificed on the 92nd day. The abstracted histories are briefly as follows:

Guinea-Pig 877.—Weighing 575 gm., was injected intraperitoneally with 2 cc of an emulsion 1:10 of the spleen of guinea-pig 807. When sacrificed on the 92nd day, the guinea-pig weighed 526 gm. and showed a nodular, enlarged spleen (weight 1.5 gm.), numerous foci in the liver, enlarged and firm lymphnodes. *B. melitensis* was isolated from the spleen, kidneys and liver. The serum agglutinated *B. melitensis* 1, 1:400 and *B. abortus* 1:400; after absorption, *B. melitensis* 27 was clumped in a dilution of 1:100, *B. abortus* <1:80.

Guinea-Pig 878.—Weighing 526 gm., was inoculated as 877. The weight at necropsy was 566 gm.; the spleen was enlarged and nodular (weight 2.1 gm.); the uterus contained 3 embryos. *B. melitensis* was isolated from the left kidney, spleen and one placenta. The serum agglutinated strains 26 and 615 in dilution >1:200 after absorption with *B. abortus* 80. *B. melitensis* was agglutinated 1:160.

Guinea-Pig 879.—Weighing 593 gm., was inoculated intratesticularly as was guinea-pig 877. At necropsy (weight 563 gm.) the nodular spleen weighed 1.2 gm., and the left seminal vesicle was distended by stringy pus. *B. melitensis* was isolated from the spleen (6 colonies), urine and left kidney. The serum reactions were as follows: *B. melitensis* 21, 1:800; No. 11, 1:400; *B. abortus*, 1:100; No. 7 and 9, <1:10; after absorption with *B. abortus*, *B. melitensis* 11 was clumped 1:640 + + +.

EXPERIMENTS WITH *B. MELITENSIS* STRAIN 6

The strain used in these experiments was obtained in 1919 from Dr. E. Sargent, Institut Pasteur d'Algerie, and was marked "Race Souk-Alvias." Six guinea-pigs were inoculated with 1/10 slant of *B. melitensis* 6. One guinea-pig (639) died on the 10th day and one (638) was sacrificed on the 15th day. Both animals showed typical lesions, while the remaining 4 guinea-pigs, when sacrificed on the

140-160th day after the inoculation, exhibited normal necropsy findings. The necropsy findings of guinea-pigs 638 and 639 are detailed.

Guinea-Pig 638.—Weighing 675 gm., was inoculated into the right testicle with 1/20 slant of a young culture of *B. melitensis* 6. It was chloroformed 15 days after the injection, when it weighed 714 gm. Necropsy revealed a generalized enlargement of the lymphnodes and atrophy of the testis; the spleen weighed 0.5 gm., was light brownish and soft. *B. melitensis* was isolated from only the spleen and the right testis. The serum agglutinated *B. melitensis* 5, 1:100 + + +; while it failed to react with *B. abortus* 320.

Guinea-Pig 639.—Weighing 505 gm., was inoculated intratesticularly as was guinea-pig 638. The animal died on the 10th day (weight 460 gm.). The spleen weighed 1.4 gm., was nodular and showed numerous yellowish necrotic foci; similar focal necroses were seen in the liver and the iliac lymphnodes; the right testis was partially necrotized and deeply injected. Cultures demonstrated the fact that all the organs, bile and urine were teeming with *B. melitensis*.

EXPERIMENTS WITH *B. MELITENSIS* STRAIN 11

The strain was obtained in 1918 as *Micrococcus melitensis* 33 from the American Museum of Natural History.

Two series of (717-726 and 753-758) 5 and 6 guinea-pigs each were inoculated intraperitoneally and intratesticularly with 1/3-1/10 slant of a young agar culture. In the first series, only 2 animals, guinea-pig 720 (intratesticularly) and 724 (intraperitoneally), gave positive cultures at necropsy. In the second series, 5 of the 6 animals injected intraperitoneally died in from 4-12 days after the inoculation; they exhibited the usual lesions, the surviving animal was negative. Two guinea-pigs (753 and 755), infected by the intratesticular route, presented typical necropsy findings; one animal (754) had succumbed to an intercurrent infection; one (756) died 7 days after the injection, and 2 (757 and 758) gave negative findings. The histories of the infected guinea-pigs are detailed.

Guinea-Pig 720.—Weighing 615 gm., received intratesticularly 1/3 slant of *B. melitensis* No. 11. Skin tests were repeatedly positive. At necropsy examination (weight 870 gm.) 127 days after the inoculation, the spleen was small, weighing 0.9 gm., the right testis was hard and atrophic, numerous lymphnodes were slightly enlarged and firm; *B. melitensis* was isolated from the pus and spleen. The serum agglutinated *B. melitensis* 1:200 after absorption with *B. abortus*.

Guinea-Pig 724.—Weighing 462 gm., was inoculated as was 720. Two cutaneous tests were positive. Sacrificed on the 127th day (weight 722). The omentum was adherent to the liver and spleen, the spleen was small and weighed 0.9 gm. The precural, postmanubrial and iliac lymphnodes were firm and enlarged; the testes were atrophic; *B. melitensis* was isolated from the precural lymphnodes. The serum agglutinated *B. melitensis* 11 after absorption with *B. abortus* 1:80.

Guinea-Pig 753.—Weighing 607 gm., was injected into the right testicle with 1/10 slant of a young culture of *B. melitensis* 11. When chloroformed on the 112th day after the inoculation, it weighed 608 gm. and showed the following lesions: Spleen slightly enlarged, weight 1.4 gm., nodular; the precrucial and iliac lymphnodes were hard and slightly enlarged; both testes were atrophic. *B. melitensis* was isolated from the right testis. The serum agglutinated *B. melitensis* 1:400 and *B. abortus* 1:100.

Guinea-Pig 755.—Weighing 613 gm., was inoculated in the same manner as guinea-pig 753. When sacrificed on the 112th day, it weighed 495 gm. and exhibited typical lesions. The spleen weighed 2.1 gm. and was distinctly nodular. The serum agglutinated several strains of *B. melitensis* as follows: *B. melitensis* 11, 1:600; *B. melitensis* 7 and 9, 1:20-1:40; *B. abortus* 80, 1:200. *B. melitensis* group II was isolated from the spleen.

EXPERIMENTS WITH *B. MELITENSIS* STRAIN NO. 18

The strain used in these experiments was obtained from the Lister Institute; it was marked *M. melitensis* (Arkwright) and had been isolated in Malta in 1915 or 1916 from the blood of a patient. Two series of 7 guinea-pigs were inoculated intratesticularly. Four animals died in from 2-15 days after the inoculation and revealed at necropsy the usual lesions of a septicemia. Three animals survived and 2 (766 and 812) presented typical anatomic and cultural findings.

Guinea-Pig 766.—Weighing 460 gm., received intratesticularly 1/10 slant of *B. melitensis* 18. Skin tests were positive on the 82nd day. Necropsy examination on the 119th day revealed an emaciated body (weight 382 gm.), an enlarged, nodular spleen, weighing 1.6 gm., firm and enlarged lymph nodes, atrophic right testis and purulent seminal vesiculitis. *B. melitensis* was isolated from the pus and the spleen. The serum was lost.

Guinea-Pig 812.—Weighing 510 gm., was injected as guinea-pig 766. At necropsy (98th day, weight 490 gm.) the spleen weighed 1.8 gm., and was distinctly nodular. *B. melitensis* was isolated only from the spleen. The serum agglutinated *B. melitensis* 18, 1:200; *B. abortus* 80, 1:1000; *B. melitensis* 7 and 9, 1:60, after absorption with *B. abortus* 80. *B. melitensis* was agglutinated 1:100 and *B. abortus* 80, <1:40.

EXPERIMENTS WITH *B. MELITENSIS* 19

The strain was received from the Lister Institute; the tube was marked *M. melitensis* Eyre—milk, Malta fever.

Two series of 4 guinea-pigs each were inoculated intratesticularly. Two animals died in from 5-15 days after the inoculation. Six guinea-pigs survived and 5 presented at necropsy normal organs with negative cultural findings, while one (770) showed these lesions:

Guinea-Pig 770.—Weighing 483 gm., was inoculated with 1/10 slant of *B. melitensis* 19. Necropsy on the 110th day revealed numerous small abscesses in the right testis and epididymis and pus in the right seminal vesicle; the spleen was small (0.65 gm.); the lymphnodes were firm and slightly enlarged. *B. melitensis* was isolated only from the pus of the abscesses. The serum agglutinated *B. melitensis* 19 in a dilution 1:200.

EXPERIMENTS WITH *B. MELITENSIS* 20

The strain used in these experiments was obtained from the Lister Institute; the tube was marked *M. melitensis* Austria I. and it was stated that the culture was originally obtained from the Royal Army Medical College, Millbank, London. This strain belongs to our group 1 of the *Brucella* group and cannot be separated from the *B. abortus* group. In paper III the *melitensis* character of the organism was proved by a pathogenicity test on one monkey.

Two series of 4 guinea-pigs each were inoculated intratesticularly. One animal died on the 22nd day after the inoculation, the spleen appeared normal, but from it *B. melitensis* was isolated. Seven guinea-pigs were sacrificed in from 72-132 days after the infection; necropsy revealed no pathological changes.

EXPERIMENTS WITH *B. MELITENSIS* 21

The strain was obtained from the Lister Institute and was marked *M. melitensis* (Basset-Smith). According to a personal communication from Surgeon Rear-Admiral P. M. Basset-Smith, Royal Naval College, Greenwich S. E., this strain was isolated by him in 1904 from a blood culture of an undulant fever patient in his ward and sent to the Lister Institute in 1905. *B. melitensis* 21 belongs serologically to group 2 or the main group of the genus *Brucella*. The pathogenicity for guinea-pigs was tested on 3 series of animals. The first series of 4 guinea-pigs gave entirely negative results. The histories of the guinea-pigs used in the second series are presented:

Guinea-Pig 819.—Weighing 543 gm., was inoculated intratesticularly (right testis) with 1/10 slant of a young culture of *B. melitensis* 21. The animal was sacrificed on the 103rd day, when it weighed 445 gm. The anatomic lesions were typical; the spleen weighed 1.82 gm. (1.2 x 4.0 cm.), showed numerous grayish nodules and a deep brownish pulp; the lymphnodes were all enlarged and hard. In the kidneys a few grayish nodules were visible. The right and left testes were atrophic, the right was fibrous. *B. melitensis* was isolated from the spleen and kidneys. The serum of the guinea-pig agglutinated various strains of the *Brucella* group as follows: *B. melitensis* 21, 1:1000-2000; *B. melitensis* 7 and 9, 1:40-1:80; *B. abortus* 80, 1:800; absorbed with *B. abortus* 80, agglutinated *B. melitensis* 1:400 + + +.

Guinea-Pig 820.—Weighing 395 gm., was inoculated as guinea-pig 819; it was found dead 10 days after the inoculation (weight 254 gm.). The right testis was partially necrotic, covered with fibrin, the spleen was deep brownish, soft, slightly enlarged (0.9 gm.), and the bile was blood tinged. *B. melitensis* was isolated from the spleen, liver, urine and from the bile; innumerable colonies developed on the blood plates. No serum could be procured.

Guinea-Pig 821.—Weighing 383 gm. and treated as 819, died on the 8th day after the inoculation. The lesions and cultural findings were identical to those recorded for guinea-pig 820.

TABLE 1
EXPERIMENTS WITH *B. MELITENSIS* 21, SERIES 3

Guinea-Pig Number	Injected With	Weight		Sacrificed	Anatomic Lesions	Bacteriologic Results	Serologic Findings Serum Agglutinates
		Initial, Gm.	At Necropsy, Gm.				
883 ♂	1/25 slant	630	529	70th day	Spleen 2.1 gm., nodular and typical; lymph nodes enlarged and hard; foel in liver and lungs; testis atrophic, left adhesions microscopically typical Spleen 1.1 gm., both testis atrophic, left fibrous; lymph nodes slightly enlarged; 8-10th right and left sternochoondral junction of ribs enlarged, nodular, containing a yellowish pus-like substance Lesions as above mainly in right ribs; spleen 0.9 gm.	<i>B. melitensis</i> isolated from left femoral bonemarrow, spleen, liver and precaral lymph node	<i>B. melitensis</i> 21, 1:2000; <i>B. abortus</i> 1:80 after absorptions with No. 80 agglutinates <i>B. melitensis</i> 21, 1:640, <i>B. abortus</i> 80, <1:20
884 ♂	1/25 slant	598	546	70th day	General lymphadenopathy; abscess in right testis and purulent seminal vesiculitis, atrophy left testis; spleen 0.9 gm. Cloudy swelling in parenchymatous organs Spleen 1.4 gm.; 12th right rib enlarged periosteochondritis on left lower femur and upper end of tibia involving left knee. Lymph nodes not enlarged	<i>B. melitensis</i> isolated from two rib lesions, spleen, liver and bonemarrow sterile	<i>B. melitensis</i> 1:1000; <i>B. abortus</i> 1:200, absorbed with <i>B. abortus</i> agglutinates <i>B. melitensis</i> 1:640
885 ♂	1/25 slant	637	515	70th day		<i>B. melitensis</i> isolated from ribs only	<i>B. melitensis</i> 1:1000; <i>B. abortus</i> 80, 1:160
886 ♂	1/25 slant	728	330 D.	41st day		<i>B. melitensis</i> isolated from testis and spleen	Not tested
887 ♂	1/25 slant	783	625	70th day		<i>B. melitensis</i> isolated from spleen and rib lesion	<i>B. melitensis</i> 21, 1:400; <i>B. abortus</i> 1:400, after absorption, <i>B. melitensis</i> 21 1:300; <i>B. abortus</i> 80, <1:20
888 ♂	1/25 slant	585	595	70th day	Spleen 0.5 gm.; both testis atrophic and seminal vesicle fibrous; right and left sternochoondral rib insertion enlarged as 884	<i>B. melitensis</i> isolated from spleen and rib lesions	<i>B. melitensis</i> 21, 1:2000; <i>B. abortus</i> 80 after absorption; <i>B. melitensis</i> , >1:1280; <i>B. abortus</i> <1:20

The exceedingly interesting and important results collected from series 3 are presented in table 1.

EXPERIMENT WITH *B. MELITENSIS* 22

The strain is known as *B. paramelitensis* (Basset-Smith) originally sent to Fleet Surgeon R. W. Basset-Smith by Dr. C. Nicolle of the Institut Pasteur de Tunis, obtained from the Lister Institute. Seven guinea-pigs were tested with this strain. Four animals died in from 10-21 days showing the usual lesions of a septicemia. Only one (822) of the 3 surviving guinea-pigs presented typical lesions at necropsy, 79-120 days after the inoculation.

Guinea-Pig 822.—Weighing 685 gm., was inoculated intratesticularly with 1/10 slant of *B. melitensis* 22. The same animal gave a strongly positive skin reaction and was therefore sacrificed on the 79th day after the injection (weight 538 gm.). The spleen was enlarged and weighed 1.4 gm. (2.5 x 1.5 cm.); the lymphnodes were small but hard; the left seminal vesicle contained creamy pus, and the liver exhibited a few foci. *B. melitensis* was isolated from the urine, spleen, seminal vesicle and both kidneys. The serum gave the following serum reactions: *B. melitensis* 22, 1:800 + + +; *B. melitensis* 11, 1:100 and *B. abortus* 80, 1:800 + + +.

EXPERIMENTS WITH *B. MELITENSIS* 26

The strain was obtained from Dr. J. W. H. Eyre, Guy's Hospital, London; it had been isolated in 1918 (?) from the spleen of a case of undulant fever. The results obtained on 9 guinea-pigs are detailed:

SERIES 1.—Guinea-Pig 834.—Weighing 480 gm., was inoculated intratesticularly with 1/10 slant of *B. melitensis* 26. The animal was sacrificed on the 108th day after the inoculation (weight 355 gm.) and revealed typical lesions. The spleen weighed 1.4 gm. (0.8 x 2.2 cm.), and the lymphnodes were slightly enlarged. The right testis was atrophic and fibrous. *B. melitensis* was isolated from the spleen. The serum agglutinated the various available strains as follows: *B. melitensis* 26, 1:1000 + + +; *B. abortus* 80 and *B. melitensis* 7 and 9 in a dilution of 1:40.

Guinea-Pig 835.—Weighing 440 gm., was inoculated in the same manner as guinea-pig 834. The animal died on the 25th day after the inoculation (weight 320 gm.); the right testis contained an abscess; the regional lymphnodes were enlarged and firm; the spleen was small (0.5 gm.). *B. melitensis* was isolated from the pus of the testicular abscess, the bonemarrow, the bile and the spleen. No serum was obtained for agglutination tests.

Guinea-Pig 836.—Weighing 360 gm., was inoculated intratesticularly with 1/10 slant of *B. melitensis* 26. The animal died on the 28th day; it was emaciated and weighed 225 gm. Necropsy revealed a spleen weighing 1.3 gm., which was soft and light brown. The lymphnodes appeared normal, while the testes were atrophic; the right testis in particular was rather fibrous. The right epididymis contained creamy pus. The gallbladder was distended and contained blood tinged bile. *B. melitensis* was isolated from the spleen, epididymis, bile and left femoral bonemarrow. No serum could be procured for agglutination tests.

TABLE 2
EXPERIMENTS WITH *B. MELITENSIS* 26, SERIES 2

Guinea- pig Number	Injected With	Weight		Sacrificed	Anatomic Lesions	Bacteriologic Results	Serologic Findings Serum Agglutinates
		Initial, Gm.	At Necropsy, Gm.				
889 ♂	1/20 slant	526	405	70th day	Spleen 2.5 gm.; (3.3 × 2.2) typical lymphadenitis; atrophic testis; foci in liver	<i>B. melitensis</i> isolated from spleen and bonemarrow	<i>B. melitensis</i> 26, 1:400; <i>B. abortus</i> 80, 1:100 absorbed with <i>B. abortus</i> 80; <i>B. melitensis</i> 1:200; <i>B. abortus</i> <1:40 Not tested
890 ♂	1/20 slant	873	823	Died 13th day	Spleen pulpy, 1.4 gm.; necrosis of right testis; cloudy swelling of liver and kidneys	<i>B. melitensis</i> isolated from bonemarrow, spleen, liver, kidneys, bile,	
891 ♂	1/20 slant	463	541	78th day	Spleen 1.6 gm.; one nodule; slight lymphadenopathy; right testis atrophic	<i>B. melitensis</i> only in spleen.....	
892 ♂	1/20 slant	519	448	61st day	Spleen small, 0.8 gm.; slight enlargement of sternochondral junction of right 8th, 6th, 7th and 8th ribs; other organs normal. Right testis small focus of necrosis Hemorrhagic orchitis; intoxication	<i>B. melitensis</i> only from right testis	<i>B. melitensis</i> 1:200; <i>B. abortus</i> 80, 1:100; absorbed <1:40
893 ♂	1/20 slant	618	603	Died 6th day		<i>B. melitensis</i> isolated from spleen, liver and bile; no other organ tested	Not tested
894 ♂	1/20 slant	513	475	70th day	Spleen 2.4 gm., typical; lymphadenopathy; atrophic right testis, abscess in left epididymis; foci in liver	<i>B. melitensis</i> isolated from spleen and seminal vesicle	<i>B. melitensis</i> 26, 1:1000; <i>B. abortus</i> 80 absorbed; <i>B. melitensis</i> , 1:4000-600; <i>B. abortus</i> 80, 1:30

EXPERIMENTS WITH *B. MELITENSIS* 27

The strain used in these experiments was obtained from Dr. T. W. H. Eyre, Guy's Hospital, London; the culture had been isolated in 1917 or 1918 from goat's milk. The results obtained on 8 guinea-pigs are detailed:

SERIES 1.—Guinea-Pig 837.—Weighing 490 gm., was inoculated intratesticularly with 1/10 slant of *B. melitensis* 27. The animal died on the 57th day when it weighed 317 gm. The lesions were typical. *B. melitensis* was isolated from the spleen (∞ colonies), right testis and seminal vesicle and the bile.

Guinea-Pig 838.—Weighing 428 gms., was inoculated in a similar manner as guinea-pig 837. It was found dead on the 57th day, when it weighed 249 gm. The lesions resembled those recorded for guinea-pig 837. *B. melitensis* was isolated from the spleen, right testis and the bile.

Guinea-Pig 840.—Weighing 728 gm., was inoculated intratesticularly with 1/10 slant of *B. melitensis* 27. On the 76th day after the inoculation, the animal gave strongly positive skin reactions with *B. abortus* and *B. melitensis* protein. The guinea-pig was sacrificed on the 99th day, when it weighed 694 gm. A typical nodular, enlarged spleen, weighing 2.3 gm. (3.2×2.5 cm.) was found. The liver was studded with numerous grayish areas, and all the lymphnodes were slightly enlarged and firm. The right testis and seminal vesicle were atrophic. *B. melitensis* was isolated from the spleen (∞ colonies), urine and left femoral bonemarrow. The agglutination reactions with the serum were as follows: *B. melitensis* 27, 1:200; *B. abortus* 1:100; *B. melitensis* 7 and 9, 1:20-40; after absorption with *B. abortus* 80, the serum agglutinated *B. melitensis* 1:100.

Guinea-Pig 881.—Was inoculated intraperitoneally with 2 cc of a spleen emulsion of guinea-pig 840. When sacrificed on the 103rd day, the normal spleen weighed 0.91 gm.; the lymphnodes were slightly enlarged, and the uterus presented an endometritis. *B. melitensis* was isolated from the spleen (31 colonies). The serum agglutinated *B. melitensis* 11, 1:400, after absorption 1:160.

EXPERIMENTS WITH *B. MELITENSIS* 29

The strain was obtained in 1919 from Dr. Guido Isar, Catania, Sicily, and was originally isolated from a case of human undulant fever. Five guinea-pigs were inoculated intratesticularly. Four animals were found free from anatomic lesions and gave negative agglutination reactions when sacrificed 72 days after the inoculation. The history of one animal (1001) is presented:

Guinea-Pig 1001.—Weighing 515 gm., was injected into the right testicle with 1/5 slant of *B. melitensis* 29. The animal presented a rough coat and gave a positive skin reaction. It was sacrificed on the 72nd day after the inoculation, when it weighed 515 gm. Necropsy revealed a small spleen (0.62 gm.); enlarged and firm precrural, iliac and peri-aortic lymphnodes (size of small peas); the left seminal vesicle contained watery pus; the right testis was atrophic and fibrous; the right radiocarpal region was enlarged and showed the typical fibrous thickening about the joint. *B. melitensis* was isolated from the urine, seminal vesicle, spleen and radiocarpal region. The serum agglutinated *B. melitensis* 29, 1:400; *B. abortus* 80.

TABLE 3
EXPERIMENTS WITH *B. MELITENSIS* 27, SERIES 2

Guinea-Pig Number	Injected With	Weight		Sacrificed	Anatomic Lesions	Bacteriologic Results	Serologic Findings Serum Agglutinates
		Initial, Gm.	At Necropsy, Gm.				
895	1/25 slant	363	270	Died 10th day	Lung edema, hemorrhagic orbit's.	<i>B. melitensis</i> from spleen, testis, but not heart blood	Not tested
896	1/25 slant	389	470	70th day	Spleen 1.8 gm.; distinctly nodular several lymph nodes enlarged. Testis atrophic	Few <i>B. melitensis</i> from spleen...	<i>B. melitensis</i> 1:400; <i>B. abortus</i> 80:1:200 absorbed; <i>B. melitensis</i> 1:100; <i>B. abortus</i> <1:40
897	1/25 slant	424	507	70th day	Spleen 1.2 (3.2 x 1.8 cm.) two nodules; lymph nodes enlarged	Few <i>B. melitensis</i> colonies from spleen	<i>B. melitensis</i> 27: 1:600; <i>B. abortus</i> 80: 1:400; absorbed: <i>B. melitensis</i> 1:100; <i>B. abortus</i> <1:40
898	1/25 slant	443	280	Died 9th day	Right testis necrotic; numerous foci in liver and spleen. Intoxication	<i>B. melitensis</i> from spleen, liver and testis	Not tested

The essential facts dealing with the number of guinea-pigs used for each strain, the course of the infection, the anatomic lesions noted at necropsy and the cultural and serologic results are summarized in table 4. It is shown that 12 strains of *B. melitensis* inoculated by the intratesticular or intraperitoneal route in amounts varying from 2,000-3,000 millions failed to produce a definite disease, which could be identified either by anatomic lesions or by positive cultures. On the other hand, 4 cultures (11, 21, 26 and 27) which were of human or caprine origin proved highly pathogenic for guinea-pigs, while 6 other strains (6, 19, 18, 20, 22 and 29) and a mixture of 5 strains (2, 3, 4, 5 and 11) occasionally caused lesions.

TABLE 4
SUMMARY OF EXPERIMENTS WITH STRAINS OF *B. MELITENSIS*

Strains	Number of Guinea-Pig Inoculated	Number of Infections	Course of Infection		Anatomic Lesions						Cultures	Serum Reactions
			Acute and Fatal	Sub-acute and Chronic	Lymph-nodes	Spleen	Liver	Lung	Testis Abscesses	Bones		
1, 2, 3, 4, 5, 7, 8, 9, 10, 23, 24 and 25...	60	0	—	—	—	—	—	—	—	—	—	—
2, 3, 4, 5 and 11.....	41	21	7	14 (44-155)	12	14	9	1	3	—	21	14
6 (Algiers human ?)...	6	2	2	—	2	2	2	—	—	—	2	+2
11 (English human 1896)	31	11	6	5	4	2	1	0	1	0	11	+4
18 (England human 1915)	7	6	4	2	1	2	0	0	1	0	6	+2
19 (England goat).....	8	3	2	1	0	0	0	0	1	0	3	1
20 (Austria human ?)...	8	1	1	—	—	—	—	—	—	—	1	—
21 (England human 1904)	13	9	2	7	5	5	0	1	2	4	9	7
22 (Para melitensis).....	7	5	4	1	1	—	1	—	1	—	5	1
26 (England human 1918)	9	9	2	7	2	4	2	—	4	1	9	5
27 (England goat 1917)...	8	8	2	6	6	6	1	0	(2)	0	8	4
29 (Sicily human ?)...	5	1	0	1	1	0	0	0	1	1	1	—
	203	76	32	44	34	35	16	2	14 (2)	6	76	40

SYMPTOMS AND COURSE OF INFECTION

The testicle, which had been inoculated with the bacterial suspension, became quickly swollen and tender, and the scrotum developed a purple reddish color. In a few animals, local ulcerations were noted. As a rule, the guinea-pigs refused their food for several days, were visibly sick and lost from 100-200 gm. of weight. The temperature was

elevated in most instances and varied between 38-40.2 C. Muscular weakness, ruffled coat and tonic spasms usually preceded death from an acute infection. The train of symptoms and the postmortem findings in the 32 instances differed in no way from those described by Eyre,⁸ following intracranial injections. Severe intoxications were common in animals infected by the intraperitoneal route. Thirty-two of the 76 successfully infected guinea-pigs died in from 2-20 days. As the experiments reported in this paper were primarily undertaken for the study of subacute and chronic lesions similar to those seen in guinea-pigs infected with *B. abortus*, all the animals which died in the first 20 days after the injection presented lesions of relatively little interest and are in this presentation classified as having acute, fatal infections. Guinea-pigs suffering from subacute or chronic *B. melitensis* infections manifested in the first few days after the injection certain of the symptoms already described. The loss in weight was distinct, and later in the course of the disease a rough coated appearance was frequently present, particularly in animals with marked focal lesions. Swelling of the radiocarpal and knee joints was observed in a series of guinea-pigs inoculated with strain 21 and in one animal infected with strain 29.

As regards the course of the disease, the animals divided themselves into 4 groups. Some ran a gradually downward course, became much emaciated and died between the 25th and 50th day after the injection. Larger animals regained their loss slowly and, when sacrificed on the 79th to the 119th day after the injection of 1/10-1/25th of a slant, had nearly reached the original weight, while others registered a loss of from 18-179 gm. In a small number of guinea-pigs, the normal physiologic increase in weight continued, and the animals were disposed of at the end of from 70 to 155 days, in excellent condition. The course of the infection was acute and fatal in 42% and subacute or chronic in 58% of the successfully infected guinea-pigs. Only 76 of the 143 animals infected with 10 different strains presented evidence, either in the form of positive skin tests or in macroscopic and microscopic lesions and positive cultures, which could be considered indicative of an infection. It is evident from these results that certain guinea-pigs have a greater resistance to *B. melitensis* than others; in fact, some of the experiments even suggested that the animals procured from certain breeders were nonsusceptible to a *B. melitensis* infection. This is the only apparent explanation which can be offered for the many failures which were obtained in several experimental series. In connection with

the experiments on *B. abortus*, more attention will be paid to this phase of the problem.

Of the 44 animals with chronic infections, 7, or 15.9%, died. Death was due to anaphylaxis in one, enteritis in another, and in the remaining 5 it followed emaciation and chronic intoxication.

PATHOLOGIC ANATOMY

The 32 animals which died in from 2-20 days following the intratesticular or intraperitoneal injection of various strains of *B. melitensis*, revealed either a necrotizing orchitis or a localized or diffuse fibrinopurulent peritonitis. The liver, spleen and kidneys were dark and engorged with blood; as a rule, the lymphnodes were enlarged and soft. In a few instances, small scattered necroses were visible in the liver. Invariably the inoculated bacteria were isolated from every organ, occasionally also from the heart blood.

The gross pathologic changes in the 44 guinea-pigs, which lived from 47 to 155 days, were in brief:

The lymphnodes were enlarged in 34 cases, or 77.2%. In about one-half of this number, the enlargement was confined to certain groups of nodes; thus the precrural and iliac groups were often quite conspicuous in the anemic inguinal and retroperitoneal fat layers. The firm and grayish nodes varied in size from that of a small grain to that of a small split pea.

The spleen was enlarged in 35 cases, or 79.5%. It should, however, be emphasized that the organ rarely attained the dimensions which were customarily observed in guinea-pigs infected with *B. abortus*. The average weight of the spleen of 17 infected and carefully weighed guinea-pigs, which lived for 70-155 days, was 1.7 gm. (average body weight 532 gm.). The maximum weight of the spleen was 3.5 gm., for an animal of 417 gm. Not infrequently the dimensions of the organ appeared normal, and without cultural or serologic tests the existence of an infection would have been overlooked. The appearance of the surface under the capsule was always nodular and speckled with grayish foci varying considerably in size, although large whitish areas, as seen in *B. abortus* spleens, were not observed in this experimental series. When definitely enlarged, the spleen was always darker than normal and presented a moist protruding pulp, with distinct foci on section. In 9 guinea-pigs, even though the spleen presented an apparently normal appearance, definite lesions were found in the genital organs or the ribs.

The liver in 16 guinea-pigs, or 36.6%, showed scattered over the surface either a few or many grayish, pin point foci. These lesions differed in no respect from those noted in guinea-pigs infected with *B. abortus*. In 3 guinea-pigs, which died on the 25th, the 28th and the 57th day, respectively, the gall-bladder contained a small amount of blood tinged bile, which was teeming with *B. melitensis*.

Only one guinea-pig revealed grayish foci in both kidneys on stripping the capsule; the genital organs were free from suppurative process in this case. The testes of all animals injected by the intratesticular route showed changes in the form of atrophy and fibrous degeneration of the parenchyma. In 9 instances the organs contained small or large abscesses. The diseased testicle was invariably adherent to the scrotal sac. The epididymis of the testicle into which the bacterial growth had been injected presented abscesses in 2 cases, while the opposite organ was involved in one instance. The seminal vesicle of

the infected right testicle contained creamy or thin liquid pus in 6 cases. In 3 instances, the left seminal vesicle was diseased and attached to a slightly atrophic and fibrous epididymis and testicle. The injections were always made in the right testicles. A striking bilateral atrophy of the testes was recorded in 9 animals.

The placenta of a guinea-pig with a triple pregnancy showed flakes of purulent exudate, and *B. melitensis* was isolated in culture from this material. Another pig with splenic lesions exhibited a chronic endometritis, but gave only a positive culture from the spleen.

The lungs of 2 guinea-pigs, or 4.5%, showed subpleural, translucent foci in the principal lobes. Five guinea-pigs inoculated with strain 21 presented marked swellings of the osteochondral junctions of several ribs. One animal had an involvement of the knee region and another, infected with strain 29, exhibited an enlargement of the right radiocarpal region. The lesions were identical with those described by Fabyean¹⁴ for guinea-pigs suffering from abortion disease.

The histologic changes were, in accordance with the macroscopic changes, either extensive or slight and could sometimes be found only after careful search of numerous sections. Common to all focal lesions in the lymphnodes, spleen and liver were the nests of epithelioid and scattered giant cells, frequently accompanied by a marked connective tissue hyperplasia. The pathologic process is indistinguishable from that seen in guinea-pigs infected with *B. abortus*.

CULTURAL RESULTS

Systematic preparations of cultures disclosed an interesting distribution of *B. melitensis* in the infected guinea-pigs. Positive cultures were obtained from:

Spleen	33 animals
Testicles, abscesses in epididymis and pus in seminal vesicles	14 animals
Bonemarrow of left or right femur.....	6 animals
Kidneys and bile.....	5 animals
Precrural lymphnodes and liver.....	4 animals
Bone lesions	4 animals
Urine	3 animals
Placenta	1 animal

It will be noted that the spleen furnished positive cultures more frequently than any other organ. In this connection, it should be stated that the number of colonies which grew on the blood plates were in many cases less than 10. Such cultures were often obtained from animals which were sacrificed after a period of observation of more than 100 days. When *B. melitensis* could not be isolated from the spleen, either the suppurative processes in the genital organs or the bonemarrow and lymphnodes furnished positive cultures. The urine gave cultures of *B. melitensis* only in 3 instances. These findings are in contrast to those of Durham, of Eyre and of Nicolle and Conseil, who obtained a fairly good growth from the secretion in about 50% of the animals dying from a chronic infection. The cultures procured from the tissues were readily classified by agglutination tests, and, as far as these methods permit correct deductions, it can be concluded that only descendants of the injected strains were recovered. One culture obtained from guinea-pig 655 was tested on a monkey and proved to be a typical strain of *B. melitensis*.

¹⁴ Jour. Med. Res., 1912, 26, p. 441.

SEROLOGIC RESULTS

The experiments dealing with this phase of the problem have been reported in paper II of this series. The serum of the infected guinea-pigs agglutinated in a dilution of 1:200-1:2,000 those cultures belonging to the same group of *B. melitensis* as that used for the infection. As a rule, the reactions were distinct and of diagnostic value. Animals exhibiting no gross lesions and no positive cultures failed to agglutinate either *B. melitensis* or *B. abortus* in dilutions above 1:40. Serum tests may give results indicative of an infection, if performed shortly after specific antigens have been used for skin tests. Most of the serums agglutinated *B. abortus*; in fact, in some instances the latter organism was clumped in higher dilutions than the infecting *B. melitensis* strain. Absorption tests, however, invariably removed the coagglutinins and thus established the true nature of the infection.

GENERAL AND LOCAL HYPERSENSITIVENESS

The intracutaneous injection of bacterial proteins prepared from *B. abortus* and *B. melitensis* cultures produced in the infected guinea-pigs marked skin reactions; in one animal (655) death followed the introduction of the protein preparations in less than 24 hours. Anaphylactic symptoms were noted and lesions of an acute intoxication were present in the form of congestion and small hemorrhages in the viscera.

It was impossible to diagnose the type of infection with the aid of the skin tests. "Aborto" as well as "melitensis protein" produced indurated areolae varying in size from 1.4-1.8 cm., although the existence of disease was definitely indicated in the animals which were not emaciated. Guinea-pigs with local suppurative processes in the seminal vesicles or testicles responded by small indurations; while generalized infections were conducive to large and pronounced skin reactions. Cutaneous hypersensitiveness tests have proved invaluable in segregating the experimental animals. The method deserves more frequent application than it has received hitherto.

SUMMARY

The data presented in the preceding paragraphs indicate that some strains of *B. melitensis* are capable of causing lesions in guinea-pigs, which are sometimes remarkably similar to those produced by *B. abortus* in this species of animals. Four cultures, 2 old strains isolated from human cases of undulant fever in 1896 and 1904; 1 human strain isolated in 1915 and 1 caprine strain recovered from goat's milk in 1917 infected guinea-pigs fairly regularly, while 18 other strains either proved nonpathogenic or occasionally produced lesions in the spleen, lymphnodes, etc. It is noteworthy that strain 20, which, morphologically, biochemically and serologically cannot be separated from the *B. abortus* group, caused acute infections, but thus far no chronic lesions have been observed. The pathogenicity of *B. melitensis* strains for guinea-pigs is influenced neither by the origin nor the age of the culture. The experiences collected in this paper suggest that the capacity of this organism to infect guinea-pigs is a property possessed by only a few strains, although the observations of previous workers strongly indicate

that recently isolated cultures or body fluids containing *B. melitensis* will usually produce distinct lesions. Individual susceptibility of the guinea-pigs may be an important factor. On 2 occasions, only 1 guinea-pig of 8 injected with cultures which had been recently isolated from definite lesions developed a chronic infection, while animals procured from another breeder and inoculated with the same strain became diseased and exhibited interesting rib lesions in 5 out of 6 cases. The tissues most frequently attacked are the spleen and lymphnodes. Lesions in the liver, lungs, kidneys and bones have also been observed. It should be emphasized, however, that the pathologic processes in the spleen and lymphnodes of *B. melitensis* guinea-pigs appear less striking than those seen in this laboratory in animals infected with *B. abortus*. In fact, the impression has been gained that *B. melitensis* strains are less invasive for guinea-pigs than *B. abortus*. It is also recalled that the first series of intraperitoneal infections showed at necropsy perfectly normal viscera, which negative results prompted subsequent use of the intratesticular route of inoculation. Sufficient data are now available to justify the conclusions that in all probability injection by the intraperitoneal or subcutaneous route, or even feeding infections, may cause specific and characteristic lesions, provided certain strains and susceptible guinea-pigs are chosen. Recently isolated strains and particularly cultures or milk specimens procured from infected goats should be tested on guinea-pigs to determine their pathogenic properties.

EXPERIMENTS WITH *B. ABORTUS*

Since Theobald Smith and Marshall Fabyean¹⁵ called attention to the lesions produced in guinea-pigs after injections with *B. abortus*, several workers have studied this interesting inoculation disease. The publications of Fabyean,¹⁴ Schroeder and Cotton,¹⁶ Fleischner and Meyer¹⁷ and Smillie¹⁸ recorded definite gross changes in the viscera, enlarged, nodular spleens, lymphadenopathy, atrophy of the testicles, induration and suppuration of the epididymis, minute foci in the liver, etc. In the majority of instances, these lesions followed the injection of milk, infected cotyledons or cultures recently isolated from diseased guinea-pigs. After careful perusal of the American reports, one cannot escape the impression that splenic lesions are always present and that positive infections occur with remarkable regularity, provided the material inoculated contains a sufficiently large number of abortion bacilli. Many years ago one of us (K. F. M.), experimenting with old stock cultures of *B. abortus*, repeatedly failed to infect guinea-pigs, and for this reason the literature was carefully scrutinized for information which might explain the failures. As some of these references are nearly inaccessible, an attempt is here made to review this data.

¹⁵ Centralbl. f. Bakteriöl., 1912, I, 61, p. 549.

¹⁶ Twenty-Eighth Annual Report Bureau of Animal Industry, 1911, p. 139.

¹⁷ Amer. Jour. Dis. Child., 1917, 14, p. 157.

¹⁸ Jour. Exper. Med., 1918, 28, p. 585.

T. C. Evans,¹⁹ of Canada, described in 1914 the lesions occurring after the inoculation of guinea-pigs with infected milk. His description agrees with that of Schroeder and Cotton, with the reservation that the lesions he found were less pronounced. He ascribed the differences to lack of resistance of his guinea-pigs on account of the cold quarters in which they were kept, or the more marked virulence of the Canadian strains. Most of his guinea-pigs died from the infection, which must, therefore, have been virulent, as most workers have found that death is not a usual sequel.

Krage²⁰ reported, in 1913, that he had inoculated 36 guinea-pigs subcutaneously, intraperitoneally and intramuscularly with 0.5-2.0 cc of a culture of *B. abortus*, which was virulent for mice. One animal died from peritonitis; 8 succumbed to intercurrent infections. The remainder were sacrificed at the end of 8 months following the infection. No macroscopic lesions were found, but the serum of the animals agglutinated *B. abortus* in a dilution of 1:1,000. In another series of 16 guinea-pigs, he injected 4 strains, highly virulent for mice, subcutaneously and intraperitoneally. Three animals died on the second day and 3 during the second and third month; one presented a hemorrhagic metritis. The remaining 10 were sacrificed at the end of 6 months. *B. abortus* was isolated from the precaval lymphnode of an intraperitoneally injected animal; the serum agglutinated *B. abortus* in a dilution 1:4,000. Two other guinea-pigs gave positive cultures from subcutaneous abscesses; one of these animals presented a slightly enlarged spleen. The serum of both guinea-pigs agglutinated *B. abortus* in a dilution of 1:100-1:400. The serum agglutination titer of the 7 guinea-pigs, which furnished no cultures, varied from 1:40-1:200.

In a third series, he injected subcutaneously and intraperitoneally the milk of goats, which had been artificially infected and which contained culturally *B. abortus*. Two died on the fourth day from peritonitis; 10 succumbed to intercurrent infections and only 4 of the remaining 26, at the end of from 5-6 months, gave serum reactions from 1:100-1:400. Tissue cultures were entirely negative.

Thomsen²¹ reviewed, in 1915, the observations which had been made by Holth, in C. O. Jensen's laboratory in Copenhagen. In 1912, Holth injected subcutaneously guinea-pigs, with 1 and 2.5 cc. of an agar slant of recently isolated strains of *B. abortus*. Two animals, sacrificed on the 44th day after inoculation, presented subcutaneous abscesses, which contained bacterioscopically and culturally *B. abortus* in pure form. One animal presented an enlarged nodular spleen and typical lesions in the ribs, while the viscera of the other guinea-pig appeared normal. The third animal, which died from pneumonia on the 264th day, and the fourth, sacrificed on the 296th day, revealed normal organs at necropsy. In a second series, he injected into 2 guinea-pigs $\frac{1}{2}$ of an agar slant of an old laboratory strain subcutaneously and intraperitoneally; a third animal received $\frac{1}{4}$ of a slant intraperitoneally. The first guinea-pig died with a loss in weight of 90 gm. at the end of 14 days and exhibited numerous abscesses in the peritoneal cavity, testicles, etc.; the second guinea-pig died on the 11th day with a loss of 190 gm. in weight; it had diarrhea, an enlarged spleen and testicles; a pronounced infiltration was noted at the site of the inoculation. The third guinea-pig died on the 36th day in an extremely emaciated state (loss in weight 320

¹⁹ Report of the Director General for the Canadian Veterinary Service, 1914. No. 5 and No. 6, Reports of the Director of Veterinary Research, Union of South Africa, Pretoria, 1919, p. 357.

²⁰ Centralbl. f. Bakteriöl., Ref., 1913, 57, p. 304.

²¹ Maanedsskrift for Dyrlaeger, 1915, 27, pp. 13 and 16.

gm.); necropsy revealed abscesses in the omentum, on the surface of the liver and in the testicles; numerous ribs and both carpal joints showed typical swellings.

Thomsen, in 1912, inoculated 13 guinea-pigs with fresh milk procured from cows which had aborted and which also gave positive serum reactions for *B. abortus*. Two guinea-pigs of this series gave cultures for *B. abortus* from a subcutaneous abscess in one and a splenic nodule in another.

In 1919, Robinson,²² in South Africa, detailed his studies on about 200 guinea-pigs, which were inoculated with infected milk or other material and killed at different periods after inoculation, varying from 4 weeks to 7 months. He found that only an enlargement of the spleen occurred with any degree of constancy—liver lesions were seen in only 3 cases. No changes were noted in the bones. Furthermore, Robinson concluded that the agglutination test applied to the serums of infected guinea-pigs is of very great use, particularly when the spleen is only slightly, if at all, enlarged and not abnormal in appearance. In his experience it seemed advisable to determine the question of infection in guinea-pigs by a combined study of the macroscopic lesions, the histologic examination and the agglutination test.

Zeller²³ injected guinea-pigs and rabbits intravenously, intraperitoneally and subcutaneously with large doses of *B. abortus* and *B. melitensis*. The necropsy findings of the animals sacrificed after varying time intervals were mostly negative. In a few cases, an enlargement of the spleen and mesenteric lymph-nodes was noted, and the injected bacteria were isolated from the spleen, uterus, testicles, urine, milk and, occasionally, the kidneys.

The findings of Krage, Holth and Zeller deserve consideration in a comparative study of the pathogenicity of *B. abortus* and *B. melitensis*. In fact, their results may explain some of the failures to produce gross lesions in guinea-pigs injected with *B. melitensis* cultures. Sufficient data had been collected in this laboratory to confirm the work of T. Smith and Fabyean, Schroeder and Cotton and others on *B. abortus* infections following the injection of infected milk or tissues of diseased guinea-pigs. Little was known, however, with regard to the effect of injecting old and recent stock cultures. According to the reports of T. Smith and Fabyean, cultures appear equally as virulent for guinea-pigs as infected material derived from cattle. Their tests were limited, however, to 2 old stock cultures, using 11 guinea-pigs. For comparison, these observations were not considered sufficiently comprehensive and a series of experiments was therefore undertaken (1) to compare the pathogenicity of old *B. abortus* stock cultures with that of *B. melitensis* cultures when applied by the subcutaneous or by the intratesticular route; (2) to study the lesions produced by recently isolated strains, and (3) to correlate the data collected from studies I and II with the findings on guinea-pigs which had been

²² No. 5 and No. 6, Reports of the Director of Veterinary Research, Union of South Africa, Pretoria, 1919, p. 357.

²³ Berl. tierärztl. Wchuschr., 1920, 36, p. 345.

TABLE 5

EXPERIMENTS WITH *B. ABORTUS* 80, SERIES 3

Inea-Pig number	Injected With	Weight		Sacrificed	Anatomic Lesions	Bacteriologic Results	Serologic Findings
		Initial, Gm.	At Necropsy, Gm.				
630	1/10 slant	722	642	15th day (moribund)	Lymphadenitis; splenic tumor 2.2 gm.; foci in liver, orchitis	Spleen, liver, testis gave positive cultures	<i>B. abortus</i> , 1:400; <i>B. melitensis</i> 11, 1:80
631	1/10 slant	730	533	23d day (moribund)	Spleen 3.1 gm.; lymphadenitis; foci in liver and lungs, cholecystitis, lesions in numerous ribs; conjunctivitis and opaque cornea	Spleen, bile, liver, urine, lymph-nodes, bonenarrow	<i>B. abortus</i> , 1:2000; <i>B. melitensis</i> 5, 1:1000
632	1/10 slant	740	547	44th day, died in hypersensitiveness test 90th day	Typical lesions; spleen 4.5 gm.	Spleen, liver, but not blood.....	Not tested
633	1/10 slant	709	465		Spleen 0.6 gm.; one rib junction right side enlarged; slight lymphadenitis; testes atrophic	Only rib lesion.....	<i>B. abortus</i> 80, 1:200; <i>B. melitensis</i> 11, 1:200
634	1/10 slant	825	797	183d day, negative skin reaction	Spleen small, 0.5 gm.; right testis atrophic	Sterile organs	<i>B. abortus</i> , 1:40; <i>B. melitensis</i> , <1:10
635	1/10 slant	600	292	Died 24th day	Toxemia; spleen, 1.4 gm.; lymph-nodes hemorrhagic	Every organ, except heart blood	Not tested

injected with milk or other infected material. These experiments are briefly described in subsequent paragraphs.

INTRATESTICULAR AND SUBCUTANEOUS INOCULATIONS OF STOCK CULTURES OF *B. ABORTUS*

EXPERIMENTS WITH *B. ABORTUS* 80

(A) Six guinea-pigs were inoculated intratesticularly with 0.1 c.c. of an agar slant of a young culture of *B. abortus* 80; the strain was originally isolated from certified milk and was about 2 years old. The results obtained in this experiment are shown in table 5.

(B) Four guinea-pigs were inoculated subcutaneously with the same amount and suspension of *B. abortus* 80, as series A. The results were briefly as follows:

Guinea-Pig 802.—Weighing originally 580 gm., gave positive skin tests and, when sacrificed on the 87th day, weighed 570 gm.; the spleen (3.5 gm.) was nodular; the lymphnodes were small, but firm; few foci were noted in the liver. The cultures made from the spleen and liver were negative, but the serum agglutinated *B. abortus* in a dilution of 1:1,000.

Guinea-Pig 803.—Weighing 612 gm.; the skin tests were positive. When sacrificed on the 87th day, the animal weighed 512 gm. and had a small spleen, 0.9 gm.; the lymphnodes were small and soft. *B. abortus* was isolated from a small abscess in the right uterine horn and the lateral ligament. The blood serum agglutinated *B. abortus* in a dilution of 1:600.

Guinea-Pig 804.—Weighing 511 gm., and gave positive skin reactions. It was sacrificed on the 87th day, when it weighed 485 gm. The spleen was small and weighed 1.2 gm. All the organs appeared normal, and the cultures remained sterile. The serum agglutinated *B. abortus* in a dilution of 1:1,000.

Guinea-Pig 805.—Weighing 570 gm. and, when sacrificed, 521 gm. (87th day). The spleen was small, 0.6 gm. From the uterus, showing an endometritis with a retained, necrotized placenta, *B. abortus* was isolated. The serum agglutinated *B. abortus* in a dilution of 1:2,000.

EXPERIMENTS WITH *B. ABORTUS* 38

The strain was obtained from the Laboratory of the Pennsylvania State Live Stock Sanitary Board; nothing definite is known regarding its origin. Six guinea-pigs were inoculated intratesticularly with 1/5 slant of a young agar culture. The results are presented in table 6.

EXPERIMENTS WITH *B. ABORTUS* 14

The strain was a stock culture originally obtained from Dr. A. Eichhorn, who had isolated the culture from the uterine exudate of an artificially infected cow. Three guinea-pigs were inoculated intratesticularly with 1/5 slant of a young culture. Two animals (696 and 697) were repeatedly tested with "aborto"-protein, but no reactions were obtained. Both guinea-pigs gained in weight and when sacrificed

TABLE 6
EXPERIMENTS WITH *B. ABORTUS* 38, SERIES 1 *

Guinea-Pig Number	Injected With	Weight		Sacrificed	Anatomic Lesions	Bacteriologic Results	Serologic Findings
		Initial, Gm.	At Necropsy, Gm.				
679	1/5 slant	249	190	15th day, died	Abscess in right testis; general fibrinous peritonitis	Testis and peritoneal fluid.....	Not tested
680	1/5 slant	269	353	74th day, positive skin reaction	Typical abortion disease; spleen 2.4 gm.; foci in liver and kidneys	Spleen, liver, kidneys, urine, positive	<i>B. abortus</i> , 1:1000; <i>B. melitensis</i> 1:1,000
681	1/5 slant	245	190	Died, 7th day	Hemorrhagic orchitis, splenic tumor, toxemia (?)	Spleen positive; other organs not tested	Not tested
682	1/5 slant	246	248	Died, 54th day positive skin reaction	General lymphadenitis; spleen small (0.6 gm.); other tissues negative	Spleen only tested and found sterile	Not tested
683	1/5 slant	305	420	95th day, positive skin reaction	Spleen small (0.8 gm.); lymphnodes firm, but small; testes atrophic	Spleen ∞ growth.....	<i>B. abortus</i> 80, 1:800
684	1/5 slant	395	267	19th day (moribund)	Necrotized right testis; spleen 0.4 gm.; slight lymphadenitis	Testis, extensive growth.....	Not tested

* Two guinea-pigs—701 and 702—inoculated intratesticularly with an emulsion of the testis of guinea-pig 684 developed abortion disease with slight lesions, positive skin reaction, but negative spleen cultures.

on the 99th day presented normal and sterile organs. One guinea-pig (695) became infected. The history is given.

Guinea-Pig 695.—Weighing 460 gm., received intratesticularly 1/5 slant of *B. abortus* 14. Skin tests on the 44th day were positive. At necropsy (weight 292 gm.) on the 50th day, the spleen was small (0.7 gm.); the lymphnodes were soft and small. The right testis contained an abscess the size of a pea and the left epididymis exhibited several small abscesses. *B. abortus* was isolated from the pus of the abscesses, but not from the spleen, liver or urine. The serum agglutinated *B. abortus* 80 in a dilution of 1:2,000 and *B. melitensis* 11, 1:800.

EXPERIMENTS WITH *B. ABORTUS* 1

The culture was an old stock culture and had originally been obtained from Sir S. Stockman, who had isolated the strain from the uterine exudate of an artificially infected cow. Three guinea-pigs were inoculated intratesticularly with 1/5 slant of a young culture. Two animals (699 and 700) gave negative skin reactions and, when sacrificed on the 86th day, presented normal and sterile organs, while, according to the history given, one guinea-pig (698) became infected.

Guinea-Pig 698.—Weighing 565 gm., received intratesticularly 1/5 slant of *B. abortus* 1. Skin tests on the 44th and 66th day were positive. The guinea-pig was chloroformed on the 86th day after the inoculation, when it weighed 570 gm. The spleen was slightly enlarged (weight 1.3 gm.) and nodular; the liver showed a few scars, and the testes were atrophic. *B. abortus* was isolated from the kidneys, but not from the spleen, bonemarrow, liver, etc. The serum agglutinated *B. abortus* 80, 1:800.

These experiments indicate that intratesticular injections of *B. abortus* may produce an acute and fatal disease. Extreme emaciation and exhaustion combined with extensive inflammatory processes are probably the main causes of death. Every guinea-pig injected with strain 38 became infected, but the anatomic changes were only typical in one animal (680). Strain 80 acted similarly; one guinea-pig presented a small spleen when sacrificed on the 90th day, but showed changes in the ribs and testicles. Another animal gave negative skin tests repeatedly and was normal at necropsy on the 183rd day. Strains 1 and 14, probably old stock cultures, are less virulent than strains 80 and 38. The animals survived the injection, but only one guinea-pig of each series gave a positive skin reaction and presented distinct anatomic lesions. As far as the pathogenicity for guinea-pigs is concerned, the effect of these 2 strains cannot be distinguished from that of typical *B. melitensis* strains. In fact, the gross lesions, the cultural findings and the serologic results were indistinguishable from those previously noted in guinea-pigs inoculated with *B. melitensis* strains 11

or 27. Subcutaneous injections of strain 80 gave positive infections, but in only 2 animals was the pathologic picture typical.

The differences in pathogenicity cannot be ascribed to the amounts of culture injected. Young cultures were suspended in saline and the turbidity of the emulsion was standardized against a typhoid vaccine containing 1 billion bacteria per c c. One tenth or $1/5$ of an agar slant of *B. abortus* contained about 1,000-2,000 million viable organisms and was approximately the same for each of the 4 strains. It is naturally impossible to make conclusive statements with regard to the virulence of a *B. abortus* strain until further knowledge relative to the individual susceptibility of the guinea-pigs has been accumulated, but the few carefully controlled observations rather forcibly suggest that old stock cultures are not only less toxic, but also less invasive than recently isolated strains or infected material derived from bovine, porcine or cavian sources. This contention is supported by some tests conducted with a strain of *B. abortus* isolated from a case of swine abortion.

INTRAPERITONEAL INJECTIONS OF *B. ABORTUS* CULTURES OF PORCINE ORIGIN

In 1920, Dr. J. Traum furnished the writers with a culture of *B. abortus* isolated from the liver of an aborted swine fetus. The strain grew profusely, in contrast with the bovine *B. abortus* strains, but could not be distinguished biochemically or serologically from the ordinary stock cultures kept in this laboratory. However, the porcine *B. abortus* manifested noteworthy invasive properties for monkeys. These facts have already been noted in papers I, II and III of this series. Twenty-four guinea-pigs were inoculated with $1/20$ slant of a vigorously growing agar culture of the second generation.

The clinical and necropsy findings are summarized in table 7.

The data presented in table 7 proved the highly virulent character of a porcine strain of *B. abortus*. Six animals succumbed to the infection in from 17-40 days after the intraperitoneal injection. The loss in weight varied from 73.5 to 345 gm. and only 6 of the 24 animals showed a gain of 10 to 195 gm., or an average increase of 41 gm. The strain exhibited pyogenic properties exemplified by abscess formation in the peritoneal cavity, omentum and lymphnodes, likewise in some instances in the spleen and liver. The testicles, epididymis and seminal vesicles of all the animals used in the test were destroyed by suppurative processes. The spleen was usually enlarged, but 2 animals presented, at least macroscopically, a normal splenic capsule and pulp. Nine

animals had involvements of the ribs and long bones, while 11 showed lesions only in the radiocarpal and tibiotarsal regions. The lungs and kidneys were never diseased. *B. abortus* was readily isolated from the spleen, testicles, etc., and the blood serum agglutinated the representatives of the *Brucella* group in dilution of 1:800-1:2,000.

Constant infections with definite localization of the lesions in the sex organs, bone structures, spleen and lymphnodes were not observed with an inoculation of less than 2,000 million organisms. Guinea-pigs purposely procured from several breeders were employed, and the striking results could therefore only be attributed to the inherent properties

TABLE 7
NECROPSY FINDINGS ON GUINEA-PIGS INOCULATED WITH *B. ABORTUS* CULTURES OF
PORCINE ORIGIN

Died or Sacrificed	Number	Average Loss or Gain in Weight, Gm.	Spleen, Average Weight	Lymph-nodes	Liver Abscesses	Abscess in Omentum	Abscess in Testes or Epididymis	Involvement of Ribs	Radiocarpal Tibiotarsal Region
Died, 17 days	2	167	1.3	Abscesses 2	0	2	1 (1 female)	0	0
Died, 18 days	1	112	0.8	Enlarged	0	1	1	1	0
Died, 20 days	1	196	0.9	Small	0	1	1	1	0
								both sides	
Died, 38-40 days	3	126	1.1	3 enlarged	1	3	2 (1 female)	2	1
Sacrificed 55 days	2	345	3.35	2 enlarged	1	2	1 (1 female)	0	0
Sacrificed 69 days	14	8-73.5 6+41.0	3.57	12 enlarged	9	8	4 (10 females neg.)	5	9
Sacrificed, 134 days	1	-238	2.21	1 enlarged	1	1	1	0	1
	24	21 enlarged	12	18	11	9	11

of the strain and not to the susceptibility of certain animals. Furthermore, F. Hayes and J. Traum²⁴ and Schroeder²⁵ have recently reported identical results, using the same and other porcine strains on guinea-pigs obtained from different sources.

INOCULATIONS OF GUINEA-PIGS WITH INFECTED MILK, GUINEA-PIG TISSUES AND CULTURES OF *B. ABORTUS*

Since 1915, the writers have been interested in the occurrence of *B. abortus* in "certified milk" and later in the problem of cutaneous hypersensitiveness in guinea-pigs infected with *B. abortus* or *B. melitensis*. In the course of these studies, several hundred male and female

²⁴ Jour. Amer. Vet. Med. Assn., 1922, 60, p. 435, and personal communication.

²⁵ Jour. Amer. Vet. Med. Assn., 1922, 60, p. 560.

guinea-pigs were inoculated with milk sediment, spleen emulsions of infected animals or cultures of *B. abortus*, etc. The necropsy examinations of 114 animals were conducted according to the principles laid down in the paragraph on methods, and the data, although originally collected for entirely different purposes, can therefore be used in a discussion dealing with the pathogenicity of *B. abortus* for guinea-pigs. For the sake of brevity results of importance are presented in tables 8 and 9.

It is to be regretted that the data summarized in tables 8 and 9 do not lend themselves to a discussion of the relative susceptibility of guinea-pigs to *B. abortus*, nor do the findings permit any conclusions relative to the virulence of the various strains employed. In the first place, it was obviously impossible to determine the number of viable organisms in the injected tissues or milk, and, secondly, in those instances in which the number of inoculated bacteria was known, namely, not more than 1,000-2,000 million bacteria per dose, it was, for various other reasons, necessary to keep the animals under observation for at least 300-400 days. As early as 1916, it was noted that a series of 48 guinea-pigs injected with old stock cultures isolated in 1910 and 1911 not only survived the infection, but 8 animals failed to give skin reactions and on necropsy were found to be free macroscopically, microscopically and culturally from *B. abortus*. At that time serum tests were not conducted systematically, and this series can therefore be considered only from the standpoint of gross lesions. The data in tables 8 and 9 have been arranged according to the material which was used for the inoculations. It is quite evident that from the tables it is possible to classify the infections as acute, subacute or chronic. The following facts are apparent:

Guinea-pigs inoculated with infected milk, cultures, etc., and permitted to live for from 29-200 days always exhibited enlarged, firm lymphnodes; the spleen was typical and enlarged (1.2 to 10.5 gm. or an average of 4.69 gm.) in 80% of the cases. Normally sized spleens were more frequently seen among females than males. Animals, which lived from 200-395 days, classified here as chronic infections, had small lymphnodes and normal spleens. Sixteen of 17 males, which came to necropsy on the 300-389th day, presented spleens of an average of 0.83 gm. In many instances, however, this organ was adherent to the abdominal wall and, on section, it was strikingly fibrous and dry. Atrophy of the testicles was always present, while abscess formation in these tissues was found in 12, or 70%, of the animals. In the acute

TABLE 8
B. ABORTUS INFECTIONS IN GUINEA-PIGS
Male and Female Guinea-Pigs Inoculated with Milk Sediment or Spleen Emulsions of Infected Guinea-Pigs

Day on Which Sacrificed	Num-ber of Guinea-Pig		Lymphnodes		Spleen		Liver		Lungs		Kidneys		Testicles ~		Uterus		Bone Lesions				Cultures from				Agglutination Titer of Serum		
			En-larged		Nor-mal								Atrophy		Ab-scusses		Prog-nancies		Ribs		Joints		Spleen			Ab-scusses	
	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.		M.	F.
29	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1
54	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
64-69	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
84-89	9	3	9	3	2	1	1	6	3	1	1	1	1	4	2	1	1	1	1	1	1	1	1	1	1	1	
94-99	14	3	14	3	2	1	1	6	3	1	1	1	1	4	2	1	1	1	1	1	1	1	1	1	1	1	
100-109	6	3	6	3	
110-119	2	2	2	2	
120-129	2	2	2	2	
130-139	3	2	3	2	
140-149	4	2	4	2	
150-159	2	2	2	2	
160-169	3	1	3	1	
170-179	3	1	3	1	
180-189	3	1	3	1	
190-199	1	1	1	1	
200-209	2	1	2	1	
50	27	50	25	..	2	40	17	10	10	12	13	4	5	1	..	3	5	1	..	39	17	5	3	54	21	..	

TABLE 9

CHRONIC B. ABORTUS INFECTIONS IN GUINEA-PIGS
Male and Female Guinea-Pigs Inoculated with B. Abortus Cultures

Day on Which Guinea-Pig sacrificed	Num-ber of Guinea-Pig		Lymphnodes				Spleen				Liver		Lungs		Kidneys		Testicles		Uterus		Bone Lesions				Cultures from				Agglutination Titer of Serum			
	En-larged		Nor-mal		En-larged		Nor-mal		F.		M.		F.		M.		Atrophy		Preg-nancies		Ab-scesses		Ribs		Joints		Spleen		Ab-scesses		>1:1000	<1:1000
	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.		
38	3	3	—	3	..	3	3	3	3	3
50-59	1	2	1	2	..	1	1	1	1	0	0
62	1	1	1	1	..	1	1	1	1	1
98	..	1	0
100	4	3	4	3	2	2	4	3	1	1	4	2	6	3
310-319	5	2	..	2	..	5	0	1	5	1	4	3	2	1	3	1	1	1	6
330-339	2	..	2	2	..	2	0	0	2	1	4	3	1	1	2	1	3	1	1	3
340-349	3	1	..	1	..	1	1	..	2	1	3	2	2	1	1	2	0	1
350-359	1	1	..	1	..	1	1	1	1	1	1	1	2	2	2	2	2
360-369	4	0	..	0	..	4	4	4	1	1	1	1	1	1	1
380-389	2	2	..	2	2	..	2	2	3	1	1	1
395	..	1	..	1	..	1	1	1	1
23	14	7	12	16	2	..	7	8	16	6	5	8	..	3	..	22	15	4	..	1	4	2	11	7	16	4	16	21	21

infections, a bilateral atrophy of the sex organs was noted in 60% and definite abscesses in 14%. Liver and lung lesions occurred more often in the course of the subacute disease; in fact, no pathologic processes in these organs were recorded in animals inoculated with cultures. Kidney lesions were rare, and involvement of the bones of the extremities was somewhat more frequently noted in the chronic than in the acute or subacute cases. Blindness was seen in 2 males suffering from a chronic infection. Isolation of *B. abortus* from the spleen was rarely successful in the chronic infections, but the pus of the focal lesions invariably contained a pure growth of the organism. The serum reactions rarely indicated the age of the disease; numerous guinea-pigs tested on the 100-150th day after inoculation agglutinated *B. abortus* in dilutions 1:200-1:400, while others living for more than 300 days clumped the organisms in dilutions as high as 1:4,000.

SUMMARY

The course and the character of the lesions produced in 160 guinea-pigs by the inoculation of 1,000-2,000 million organisms of different strains of *B. abortus* or body fluids or tissues containing this bacterium can be summarized in 3 groups, namely:

(a) Acute toxic and fatal infections frequently follow the intratesticular inoculation of certain stock cultures. In this series, they occurred also following the intraperitoneal injection of a strain of *B. abortus* isolated from an aborted swine fetus. Lesions in the spleen are slight or absent; *B. abortus* can be found in all tissues, but not in the heart blood.

(b) The classical subacute infections, as described by T. Smith and Fabyeau, with very low mortality, pronounced cutaneous hypersensitiveness, positive serum reactions and distinct lesions in the lymphnodes, spleen, liver, kidneys, lungs, bones, etc., follow quite regularly the subcutaneous, intraperitoneal or intratesticular inoculation of infected material or recently isolated cultures. Old laboratory strains may occasionally produce this form of the disease in a few guinea-pigs. The spleen is always enlarged and nodular, while the other tissues may or may not be affected. Some strains exhibit a tendency to localize in the bone structures.

(c) Chronic infections with cutaneous hypersensitiveness, marked positive serum reactions and small spleens, but definite lesions in the genital organs or bones of the extremities, are noted in guinea-pigs injected with old stock cultures. Male guinea-pigs present at necropsy

a small spleen, abscesses in the testicles, epididymis, or a suppurative seminal vesiculitis and swelling of the radiocarpal or tibiotarsal regions. Cultures can frequently be procured only from the pus of these lesions. Females may fail to show gross changes; they are never pregnant, and they may present a vaginal discharge. The spleen is always fibrous and atrophic, but may occasionally yield a positive culture. The existence of an infection can only be established by skin tests with aborto protein, by serum agglutination tests, or by careful histologic studies.

The average weight of the spleen in the various groups is as follows:

	Average Body Weight	Spleen Weight
Normal guinea-pigs (50).....	485.4 gm.	0.79 gm.
Group A—Acute and fatal (12).....	376.4 gm.	1.3 gm.
Group B—Subacute (10)	440.0 gm.	4.69 gm.
Group C—Chronic (14)	607.5 gm.	0.83 gm.
<i>B. melitensis</i> infections (17).....	532.0 gm.	1.70 gm.

The experiments reported in these paragraphs support the view that *B. abortus* may produce in guinea-pigs an inoculation disease with tissue injury of varying degree of intensity. This conclusion applies in particular to the changes in the spleen and to the infections produced with cultures. The acute infection, as a rule, extends over a relatively short period and reparative processes appear at the end of a month. These animals may recover but harbor the inoculated *B. abortus* in foci of the genital organs. This sexotropism, which follows any form of injection, is remarkably constant and is of considerable diagnostic value. Infections involving all the tissues of the body with the exception of the muscles follow the injection of a porcine strain of *B. abortus*. The enlargement of the spleen has been most striking and constant in guinea-pigs injected with body fluids or tissues infected with bovine *B. abortus*.

The observations are not sufficiently extensive to warrant final conclusions, but they indicate that old *B. abortus* cultures may either fail to cause a disease, or the anatomic lesions are so slight that they cannot be seen with the unaided eye. It is this group of cases which resembles anatomically the infections caused by the intratesticular or intraperitoneal injections of the majority of *B. melitensis* cultures.

DISCUSSION

This comparative study has clearly shown that the two organisms of the *Brucella* group are pathogenic for guinea-pigs, but *B. abortus* is,

as a rule, slightly more invasive and virulent than *B. melitensis*. As has been pointed out, this statement deserves further experimental study with *B. melitensis* strains derived from caprine or human sources. In paper III of this series of investigations, it has been shown that *B. melitensis* is far more invasive for monkeys than *B. abortus*, while the tests on guinea-pigs apparently prove the reverse. The organisms which have been tentatively placed, on account of their bacteriologic similarity, into one group, can probably in the light of the studies of this laboratory be distinguished by their serologic behavior and pathogenicity for monkeys and guinea-pigs. For the experimental pathologist this peculiar variability in the pathogenicity for laboratory animals offers many fascinating problems. The solution of the question "Can the *B. abortus* be transformed into the *B. melitensis* and vice versa?" deserves careful attention in the future. Some attempts along this line have been made without obtaining any definite leads. Furthermore, cross-immunization tests have been undertaken with equally negative results. In two series, 5 guinea-pigs were treated for several months with ascending doses of fresh heat killed cultures of *B. melitensis* 11 and 20, *B. paramelitensis* 9 and *B. abortus* 80. Ten days after the last injection, each animal, together with 5 control, normal guinea-pigs, were inoculated subcutaneously with 2,000 million *B. abortus* strain 80, or strain swine fetus. Certain normal and certain immunized animals died acutely following the injection of the porcine *B. abortus* strain, while the surviving guinea-pigs, when sacrificed on the 60th-100th day after the test inoculation, presented either moderately severe lesions when injected with *B. abortus* 80 or extensive tissue changes when tested with the highly invasive porcine strain. The vaccinated guinea-pigs exhibited the same lesions as the control animals. Studies are in progress to solve the same problems on rats and mice.

The anatomic lesions produced in guinea-pigs by the injection of *B. melitensis* deserve some consideration and may perhaps explain some of the necropsy findings reported in human and caprine cases of undulant fever. As the mortality from this disease is usually low, the number of carefully conducted human postmortems is relatively small, and the available information is correspondingly small and incomplete. However, some facts immediately attract attention: Thus, it is stated by Kennedy²⁶ that the lymphnodes are predominantly diseased, the spleen is enlarged and, according to Basset-Smith,²⁷ the latter organ

²⁶ Report Mediterranean Fever Commission, 1906, Part IV, p. 94.

²⁷ Jour. Roy. Army Med. Corps, 1908, 10, p. 70.

shows microscopically a marked increase in endothelial cells and a decrease in lymphatic tissues. Not only the mesenteric lymphnodes, but also the axillary and inguinal nodes are enlarged and contain innumerable specific organisms.

Fluctuating swellings over the sternum and ribs have sometimes been mistaken for abscesses, but no pus could be procured. In reading these descriptions, one recalls the rib lesions noted in guinea-pigs. The clinical histories of Luger,²⁸ Lombard and Bégner²⁹ and others mention epididymitis, orchitis, periostitis and mono-arthritis as metastatic processes, which are not uncommon in the course of an undulant fever attack. Eyre³⁰ records amenorrhea and occasionally abortion as sequelae of *B. melitensis* infections in women.

The Reports of the Mediterranean Commission, of Mohler and Hart³¹ and of others dealing with the necropsy findings of goats infected with *B. melitensis* record an enlarged spleen, general enlargement of the lymphnodes, distinct hyperemia of the liver and kidneys. Abortion is a frequent sequel of malta fever in goats. Furthermore, *B. melitensis* has been isolated regularly from the spleen, lymphnodes, mammary glands and occasionally from the urine. The diseased tissues have not been studied extensively. Spagnolio,³² who examined the spleens of 42 goats, noted focal nodules consisting of epithelial cells and central necroses, while Neri³³ reported the lesions of a definite mastitis. There is no doubt that *B. melitensis* possesses in man and goats, just as *B. abortus* in guinea-pigs, a predilection for the perivascular and subcapsular tissues of the spleen, lymphnodes and bone structures. The specific ubertropism of both organisms is only an expression of a marked sexotropism, which in females may lead to abortion or localization of the bacterium in the udder; while in males it manifests itself in the form of suppurative processes in the testicles, epididymis, etc. The latter fact, known for *B. melitensis* in man, has recently been recognized by Buck, Creech and Ladson³⁴ for *B. abortus* in bulls. It is evident from the observations recorded in this paper and the theoretical considerations just presented that the bacteria belonging to the *Brucella* group exert their specific pathogenic properties

²⁸ Deutsch. med. Wchnschr., 1921, 47, p. 321.

²⁹ Presse Méd., 1921, 29, p. 753.

³⁰ Handbuch d. path. Mikroorg., 1912, p. 438.

³¹ Twenty-Fifth Annual Report of the Bureau of Animal Industry, 1908, p. 279.

³² Centralbl. f. Bakteriöl., Ref., 1908, 42, p. 677.

³³ Ann. d'ig. sper., 1912, 21, p. 321.

³⁴ Jour. Agric. Res., 1919, 17, p. 239.

not only in the original host, the goat, the cow and the swine, but also in the small laboratory rodents. It is therefore not unlikely that our knowledge concerning the pathogenesis and immunity, which is intimately connected with the control and eradication of two important economic diseases, namely, infectious abortion in cattle and swine and undulant fever in goats, can probably be advanced by a careful study of the inoculation disease in guinea-pigs.

CONCLUSIONS

The experiences collected in this paper indicate that certain strains of *B. melitensis* are capable of producing an acute, subacute or chronic inoculation disease in guinea-pigs. The gross anatomic and the histologic changes resemble those commonly noted in guinea-pigs infected with *B. abortus*. In fact, it is sometimes impossible to distinguish the two infections without careful serologic cross absorption tests. Four cultures, 2 old strains isolated from human cases of undulant fever in 1896 and 1904, one human strain isolated in 1915, and one caprine strain recovered from goat's milk in 1917, infected guinea-pigs regularly, while 18 other strains proved either nonpathogenic or produced in exceptional instances lesions in the spleen and lymphnodes. Aside from the inherent pathogenic property of certain strains, it is not unlikely that the individual susceptibility of the guinea-pigs and the mode of injection are in a large degree responsible for the course and the character of the infection. Intratesticular injections have been used most frequently.

Thirty-four of 44 guinea-pigs, which were sacrificed 44-155 days after the injection with cultures, presented advanced tuberculosis-like lesions in the spleen and lymphnodes. The average weight of the diseased spleen was 1.7 gm. The largest spleen observed in these experiments weighed 3.5 gm. Localization of the disease in the liver and testicles, as well as proliferation of the bone structure, has been recorded. As a whole, the lesions produced by *B. melitensis* in guinea-pigs are less extensive and severe than those produced by *B. abortus*.

Experiments extending over several years support the well known fact that *B. abortus* may produce in guinea-pigs an interesting inoculation disease. However, the degree of tissue injury in the spleen and lymphnodes may vary considerably. Sexotropism, which follows any form of inoculation whether caused by infected material or cultures, is remarkably constant. Infections involving all the tissues of the body,

with the exception of the muscles, has been characteristic for a strain of *B. abortus* isolated from an aborted swine fetus. Enlargement of the spleen with a maximum weight of 10.5 gm. and an average weight of 4.69 gm. has been noted in guinea-pigs injected with infected milk or tissues. Old laboratory cultures, as a rule, produce lesions which are so slight that they cannot be seen with the unaided eye. This group of cases resembles anatomically the infections caused by intratesticular or intraperitoneal injections of certain *B. melitensis* cultures.

Recently isolated strains and, in particular, cultures or milk specimens procured from goats affected with malta fever should be tested for pathogenic properties on guinea-pigs.

THE SEROLOGIC RELATIONSHIPS BETWEEN STRAINS OF THE PFEIFFER BACILLUS

INFLUENZA STUDIES. X *

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The importance of determining the essential nature of the various strains of the Pfeiffer bacillus found in epidemic influenza has been clearly set forth by Park and his coworkers.¹ If the strains isolated during the 1918-1920 epidemic were of a single immunologic type and differed from those isolated during interepidemic periods the presumption that a specific strain of this organism was the cause of the pandemic would be greatly strengthened. The majority of recent investigators who have studied this question have failed to find any general antigenic relationship among the strains of epidemic origin.² Maitland and Cameron,³ in a study of 38 strains isolated from hospital patients during a period when no cases of epidemic influenza were seen, found that "nearly all strains of *B. influenzae* possess a serological individuality as determined by agglutination and agglutinin absorption." On the other hand, Huntoon and Hannum⁴ obtained "an almost complete absorption of agglutinin for immunizing and heterologous strains" and found no strains among their collection which did not show "relationship either directly or indirectly through absorption." Small and Dickson⁵ also in the study of 10 strains recognized 4 different immunologic groups, and finding that 3 fell in one group and 4 in another, state that "70 per cent. of the strains studied fell into two groups." Chesney,⁶ who studied 12 strains of hemoglobinophilic bacilli isolated from influenza patients in 1920, found evidence of a serologic relationship in 4.

Received for publication, April 15, 1922.

* This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York. Part of the expense of these studies has been met by a grant from the University of Chicago.

¹ Jour. Am. Med. Assn., 1919, 73, p. 318. Park, W. H., and Williams, A. W.: Am. Jour. Pub. Health, 1919, 34, p. 45. Valentine, E., and Cooper, G.: Jour. Immunol., 1919, 4, p. 569.

² Povitzky, Olga, and Denny, Helen: Ibid., 1921, 6, p. 65. Coca, A. F., and Kelley, Margaret: Ibid., 1921, 6, p. 87. Utheim, K.: Jour. Infect. Dis., 1920, 27, p. 460. Bell, H. H.: Ibid., p. 464. Cooke, J. V.: Ibid., p. 476. Anderson, Ruth, and Schultz, O. T.: Jour. Exper. Med., 1921, 33, p. 653. Yabe, S.: Brit. Jour. Exper. Path., 1921, 2, p. 197.

³ Brit. Jour. Exper. Path., 1921, 2, p. 283.

⁴ Jour. Immunol., 1919, 4, p. 167.

⁵ Jour. Infect. Dis., 1920, 26, p. 230.

⁶ Jour. Infect. Dis., 1921, 29, p. 132.

Our possession of a large number of Pfeiffer bacillus strains isolated at different times and places has given us a favorable opportunity for studying this question. The strains tested have comprised:

13 strains from throats of patients ill with influenza at the Great Lakes Naval Training Station, Jan., 1920.

7 strains from throats of patients ill with influenza at Camp Grant, Rockford, Ill., Jan., 1920.

11 strains from students at the University of Chicago who were suffering from rhinitis, pharyngitis or other respiratory ailment, 1919-1920.

2 strains from University of Chicago patients ill with influenza, 1918.

16 strains from persons apparently in good health and with a normal upper respiratory tract; these strains were isolated between Feb. 15, 1920, and March 31, 1920.

4 strains from patients ill with influenza in New York City, Oct. 1918; obtained through the kindness of Dr. A. W. Williams.

1 strain from sputum of patient ill with influenza, Chicago, Ill., Oct., 1918.

1 strain from lung of patient dying of influenza, Chicago, Ill., Oct., 1918.

1 strain from throat of normal person, New York City, Oct., 1918.

1 strain from cerebrospinal fluid of patient with meningitis; obtained through kindness of Dr. D. J. Davis, Chicago.

Some of these strains were obtained from one and the same individual, but each strain isolated has been kept carefully separate throughout with due precautions as to purity. The agglutinative behavior of the independent strains from a single source will be referred to later.

The method of obtaining an agglutinating serum was essentially similar to that followed by other observers. Rabbits were injected intravenously with a physiologic salt suspension of the Pfeiffer bacillus washed from 24-hour heated blood agar slants. A series of 3 daily injections was followed by a 4 day rest and then the injections were resumed, this procedure usually being repeated for a period of several weeks before a serum with sufficiently high titer was obtained. Heat-killed suspensions were used for the first of these injections. Dosage of living cultures was increased up to one full agar slant. Bleedings were made after rest periods, the blood being aspirated from the heart in amounts of 10-20 c.c. The serum was separated after the blood had stood over night.

The serum test mixtures were made by adding to 0.1 c.c. of the appropriate serum dilution, 0.9 c.c. of a light suspension of Pfeiffer bacilli, 0.8% salt solution being used for all suspensions and dilutions. These mixtures were placed in a 56° C. water bath and examined after 24 hours and again after 2 days.

It was often, but not always, true that agglutination was as far advanced after 1 day as after 2. The agglutination of the homologous strains is more rapid than that of the heterologous. While the action of agglutinative serums on the homologous strains was almost invariably complete after the first day, heterologous strains were often acted on more slowly. In one instance only about one-fourth of about 50 heterologous strains showed complete agglutination at 1:50 or higher (titer 1:1,600) after 24 hours, while five-sixths of this number showed agglutination after 2 days.

For one typical experimental series 5 serums were prepared: one from a strain isolated from human lung at necropsy (indol +), one from the meningeal fluid in a case of meningitis (indol +), one from the nasopharynx of an influenza patient (1920) (indol —), one from the nasopharynx of a presumably normal person (1920) (indol +), and one from the nasopharynx of a person with rhinitis (indol —). In general the titer of the serums tested was about 1:1,600 (complete agglutination), but in a few instances serums with a titer of 1:800-1:1,200 were used. With such strengths the majority of the heterologous strains were agglutinated completely in a 1:200 dilution, sometimes higher, and occasionally, though rarely, as high as the homologous strains. A careful study of all our tables, which it seems hardly necessary to reproduce here in detail, shows no apparent relation between the origin of the strain and the action of its agglutinogenic serum on other organisms. The strains from influenza cases were not agglutinated in larger numbers or to a greater degree by the serum from an influenza strain than were those strains obtained from normal individuals. Strains from pathologic lesions yielded serum which agglutinated strains from the nasopharynx of healthy persons no more and no less than did serum from animals inoculated with "normal" strains. Indol-producing strains gave serum which agglutinated indol-producing and nonindol-producing strains without discrimination. The reaction that was most constant was that the culture used to produce the serum would be agglutinated more rapidly and in higher dilution than any other. Occasionally, however, a heterologous strain would be agglutinated to the titer limit.

A horse was immunized with a Pfeiffer bacillus strain (07) obtained from a pneumonic lung at necropsy in the 1918 influenza epidemic. Rabbits have reacted quite sharply to this strain and immunization of these animals was effected with some difficulty. After

some trials an agglutinative rabbit serum of the average titer (1:800-1:1,600) was obtained, and comparison of the agglutinative action of rabbit and horse serums carried out. Normal horse serum seems to possess somewhat greater agglutinative powers for the Pfeiffer bacillus than does normal rabbit serum. Parallel tests on 48 strains of various origins with immune horse serum and immune rabbit serum showed no noticeable difference. The titer limit of the horse and rabbit serums in one experiment was 800. Only the homologous strain was agglutinated to the titer limit. The other results were as follows: Of 47 heterologous strains, horse serum (800) agglutinated 8 at 400 and 15 at 200 and rabbit serum (800) 7 at 400 and 14 at 200.

Spontaneous agglutination of Pfeiffer bacillus cultures has troubled some observers.⁷ This difficulty when it occurs can be largely overcome by diminishing the amount of salt in the suspending solution to 0.2%. The best means of avoiding spontaneous agglutination, however, is to keep the cultures in a condition of good vitality by frequent transfers. Stock cultures should be transferred at least twice a week and a 24-hour culture always used for testing. The following experiment shows the effect of unsuitable methods of growth on the production of spontaneous agglutination. Sixty-five strains were kept on chocolate agar at 37 C. for 7 days and then for a few days longer at room temperature. About 50 of these strains were successfully nursed back into culture, at first usually scant. These strains had in previous tests averaged about 10% spontaneous agglutination in 0.8% NaCl solution at 55-56 for 2 days. After their subjection to the unfavorable conditions mentioned over one-half and in one experiment about two-thirds of the strains agglutinated spontaneously.

While the agglutination of homologous strains with homologous serum at intervals of a few days gives uniform results, that of the heterologous strains under the same circumstances shows considerable variation. A strain which on the first test does not show agglutination at 1:50 may be agglutinated a few days later under apparently the same conditions in a 1:200 dilution, and conversely. Some examples of this irregularity in agglutination of heterologous strains appear in table 1.

If enough tests were made practically every serum would in the course of several trials agglutinate partially or completely every heterologous strain.

⁷ Wollstein, M.: Jour. Exper. Med., 1919, 30, 555; Yabe, Brit. Jour. Exp. Med., 1921, 2, p. 127.

There is no doubt that the agglutination of various strains of the Pfeiffer bacillus with the serum of rabbits immunized against a single strain betokens a definite immunologic relationship. Our observations have been controlled by other serums. Serum of normal rabbits was frequently tested and affected the suspensions slightly, when at all. Serums of animals immunized to *M. catarrhalis*, *B. typhosus*, etc., produced no agglutination of Pfeiffer bacilli beyond an occasional trace in the lowest dilution. The "partial agglutination" of the heterologous Pfeiffer bacilli with Pfeiffer bacillus serum was in every case more coarse and distinct than the heaviest clumping produced by any of the

TABLE 1
VARIATIONS IN AGGLUTINATION OF HETEROLOGOUS STRAINS *

Strain	Serum C56NP (Rhinitis Strain) (Tests Repeated at Intervals of Few Days)			
	1st	2d	3d	4th
C56NP (rhinitis) homologous strain.....	800	800	—	800
GL1Tba (influenza) heterologous strain.....	0 (50±)	0 (200±)	120	50
GL6NPob (influenza) heterologous strain.....	0 (200±)	200	240	200
GL10Tbb (influenza) heterologous strain.....	0	0 (100±)	—	50
N9NPob (normal) heterologous strain.....	0 (200±)	0	—	0
N10NPoa (normal) heterologous strain.....	0 (200±)	200	0 (240±)	800
N22NPob (normal) heterologous strain.....	—	100	0	0 (200±)
R1NPoa (influenza) heterologous strain.....	0	200	120	100

* The readings were all made after 2 days, as stated. The designation 0 signifies no agglutination at 1:50; the ± sign after numbers in parentheses partial agglutination, and the whole numbers complete agglutination at the dilution specified, 1:800, 1:200, etc.

control serums. As stated, moreover, complete agglutination of all strains of the Pfeiffer bacillus could usually be observed in dilutions 1:100-1:200 if a sufficient number of tests were made.

The immunologic relationship of the different strains is, however, not as close as that of different strains of the typhoid bacillus and some other organisms. Heterologous strains, irrespective of origin, were almost invariably agglutinated in much lower degree than the homologous. Occasionally the strains from a certain locality or more strictly from a group of more or less closely associated individuals would manifest certain immunologic resemblances. Thus the strains (13 in number) from a series of influenza cases at the Great Lakes Naval Training Station, February, 1920, although differing among themselves, as a group behaved to some extent and was agglutinated

more generally by certain serums, less generally by others. For example, the serum produced by a strain from influenzal meningitis had less effect on these 13 Great Lakes strains than on those of any other group (influenza strains from Camp Grant, from Chicago, and from New York, strains from normal persons, from persons suffering with colds, etc.). On the other hand, 9 strains isolated from the nasopharynx of normal persons at Pasadena, Calif., in 1921, showed no indication of locality grouping although the strains were obtained from the throats of students who were in almost daily contact with one another. The Pasadena strains were tested with 3 serums, one prepared with a Great Lakes influenza strain, the other 2 with a Chicago rhinitis strain, control tests were made with 9 strains from miscellaneous sources. The proportion of complete, partial and negative agglutinations was substantially the same throughout, and the Pasadena strains did not behave in any instance as an approximately homogeneous group.

The strains used in this work were derived from single colonies which were again plated out before adding the cultures to the stock. By the ordinary standards these were pure cultures. The observations we have already recorded, however, that repetitions of the agglutinative tests with the same strain and the same serum do not give identical results, suggested that perhaps the cells in a culture were not equally agglutinable. A preponderance of agglutinable or non-agglutinable races in a given culture would change the reaction correspondingly. Difficulties have been experienced by some investigators in obtaining pure cultures from material containing several strains.¹

This possible explanation of agglutinative variations was studied in some detail. Thin suspensions of Pfeiffer bacillus culture were thoroughly shaken and then smeared over heated blood agar plates. Individual well separated colonies were transferred to culture tubes and the several subcultures tested in the usual way with the serum of immunized rabbits. A serum prepared from a Great Lakes influenza strain agglutinated with great uniformity 37 homologous substrains picked from individual colonies as described. One culture which was agglutinated less strongly than the others was suspended, thoroughly shaken and plated according to the method given in the foregoing. The 33 individual substrains obtained in this way agglutinated uniformly to the titer limit of the serum (3,200).

Cultures from some of the colonies were purified yet more carefully by 3 times successive plating. Two of these were used to immunize rabbits and the serums were tested against 45 individual colony sub-strains from the original stock. Agglutination occurred uniformly in all cases at dilution of 1:1,600-1:3,200. A strain isolated from a person with a common cold and one from a normal throat were similarly examined. Both showed uniformly high agglutination in all substrains.

These results so far as they go give no ground for assuming that the variations observed in the agglutination of heterologous strains of the Pfeiffer bacillus are due to the predominance of differently agglutinable races within one culture.

A possible antigenic relationship between *B. pertussis* and the Pfeiffer bacillus is suggested by some of our tests. Five strains of *B. pertussis* from different sources agglutinated in 1:50 or 1:100 dilution with serums obtained from two different rabbits immunized against a Pfeiffer bacillus strain (influenza). The serums of 2 other rabbits, one immunized against a "normal" Pfeiffer bacillus strain, the other against a "cold" strain, failed to agglutinate the strains of *B. pertussis* (1:50). Normal rabbit serum also gave negative results. Six strains of *B. bronchisepticus* were not agglutinated by any of these serums.

Most observers concede the greater accuracy of absorption tests over direct agglutination in attempts to determine serologic relationships.

We employed the following absorption method: The serum, mixed with an equal volume of centrifugally packed bacterial mass, was diluted to 1:15 with 0.8% salt solution. This mixture was heated in a 56 C. water bath for 1 to 3 hours, being frequently shaken up during this process. A 2-day incubation period was found undesirable for absorption of agglutinins by a heavy bacterial mass since so long a period impaired the serum's reactivity (table 2) even if the bacteria employed were of a totally different species. After 1 to 3 hours' incubation and after a day's storage in the refrigerator, the mixtures were centrifuged for removal of the serum dilutions.

Absorption of agglutinins by the immunizing strain was readily demonstrated by titer determinations of these serums. The homologous strain absorbed all agglutinins, homologous and heterologous. Several experiments gave similar results, those of table 2 being representative.

Absorption of the heterologous strain agglutinins, on the other hand, could not be determined by similar titer comparisons. Such

agglutinative titers were generally very low to start with and were quite variable on repeated tests. It was, therefore, difficult to decide whether the titer was reduced or perhaps eliminated by absorption, whether there had been nonspecific absorption, or whether the readings happened to be lower on the test made with the absorbed serums. Simultaneous tests of various serums at a given titer offers control against these sources of error. The latter method proved superior for determinations of absorption by heterologous strains.

The 1:15 dilution of absorbed serum was distributed to agglutination tubes, 0.15 c c to each, and a bacterial suspension added, 0.85 c c to each. The resulting 1/100 serum dilution with bacterial suspension was heated in a 56 C. water bath and read after 24 and again after 48 hours.

Table 3, following this method, indicates that homologous and heterologous Pfeiffer bacillus agglutinins are both present to a varying

TABLE 2
SPECIFIC AND NONSPECIFIC ABSORPTION

Strains of Pfeiffer Bacillus Sources	Test Serum of Rabbits Immunized to Strain C 56 NP: Agglutination After					
	Absorption 3 Hours at 56 C. by			Absorption 2 Days at 56 C. by		
	C 56 NP	GL 1 Tba	B. Prodigious	C 56 NP	GL 1 Tba	B. Prodigious
C 56 NP (rhinitis).....	0	800	800	0	200	0 (p. 200)
GL 1 Tba (influenza).....	0	0	100	0	0	0
C 93 NP (normal).....	0	200	200	0	100	0 (p. 200)
O 7 (influenzal lung).....	0	0 (p. 100)	200	0	0	0

degree in unabsorbed immune serum. Such agglutinins are not present, beyond an occasional trace, in serum from a nonimmunized animal. A Pfeiffer bacillus strain homologous to that producing the immune serum, absorbs both homologous and, for the most part, heterologous agglutinins. A heterologous strain absorbs agglutinins homologous to itself, and to a definite though variable degree it may absorb other heterologous agglutinins. Absorption by a heterologous strain does not remove, nor even appreciably reduce, the high titer of the serum for its homologous strain (see titer comparisons given in the table).

All 56 strains of Pfeiffer bacilli were tested by the absorption method, with results not differing in any essential from those recorded in the foregoing. We have sometimes observed what is ordinarily termed an immunologic relationship between strains of different origin, as illustrated in table 3 between GLITob (influenza)

and C 218-2 NPoa (rhinitis). These strains were isolated in persons living in localities over 50 miles apart. No direct or indirect contact was known to exist, and is highly improbable. The influenza strain was isolated at the Great Lakes Naval Training Station in January, 1920, the rhinitis strain in Chicago in March of the same year; the latter is indol-negative, the former indol-positive. Whether a similarity of agglutinative behavior under such circumstances has much biologic, far less much epidemiologic, significance seems doubtful. So far as the Pfeiffer bacillus is concerned, the results obtained by the absorption method are no more illuminating than those obtained by direct agglutination. In a certain sense they even may be confusing, as in the apparent "immunologic identity" of the strain mentioned, and in the

TABLE 3
ABSORPTION OF HETEROLOGOUS STRAIN AGGLUTININS *

Source of Strains	Serum of Rabbits Immunized to Strain C 56 NP, 1:100 Dilution							Control Normal Serum	Control Suspension
	Not Absorbed	After 1½ Hours Absorption by							
		B. coli	GL 1 Tob	N 10 NPoa	C 20 NO	C 218-2 NPoa	C 56 NP		
GL 1 Tob (influenza)...	++	+++	0	++	+++	0	0	0	0
N 10 NPoa (normal)...	+	+	Tr.	0	0	Tr.	0	0	0
C 218-2 NPoa (rhinitis)...	+++	+++	0	++	+++	0	0	0	0
C 56 NP (rhinitis).....	+++	+++	+++	+++	+++	+++	0	0	0
GL 6 NPob (influenza)	++	++	Tr.	Tr.	Tr.	0	0	0	0
GL 8 NPoa (influenza)...	+++	+++	Tr.	++	++	0	0	0	0
GL 10 Tbe (influenza)...	0	0	0	0	0	0	0	0	0
N 33 NPob (normal)....	+++	+++	0	++	..	Tr.	Tr.	0	0
C 56 NP titer.....	400	400	400	400	400	400	0	0	0

* The symbols used are to be construed as complete agglutination for +++, very pronounced for ++, pronounced for +, slight or trace for Tr. and none for 0.

"immunologic independence" of 6 strains isolated from the 6 members of one family as recorded by Valentine and Cooper.¹ An interesting instance of discordant results in absorption experiments has been given by Coca and Kelley.²

We have had the same experience as other observers (Povitzky and others,² Utheim,² Maitland and Cameron³) in occasionally finding several agglutinatively different strains in the respiratory tract of one person. In one patient suffering from influenza we found three strains that were distinct by the agglutinin absorption test. Bell² has reported a similar instance. Such findings are, to be sure, exceptional. We have usually found that when several colonies of the Pfeiffer bacillus are picked from a plate made from a throat swab they are of a single "immunologic" as well as cultural type.

The phenomena observed by ourselves and others in studying the agglutinative reactions of different strains of the Pfeiffer bacillus with different immune serums may be interpreted in a relatively simple way. If we assume that the Pfeiffer bacillus represents a group of semi-parasitic or commensal micro-organisms with a predilection for inhabiting the human throat and with relatively slight invasive power we should have reason from analogy to suppose that agglutinative uniformity would be improbable. It is well known that the high agglutinative homogeneity exhibited by various strains of the typhoid bacillus is exceptional among bacterial species. The group of pneumococci comprises three or more definite or fixed types, and a large number of other strains possessed of more or less independent qualities. Pfeiffer bacilli apparently stand yet farther away from a definitely established group-pathogenicity although their invasive power under certain conditions is undoubted. The presence of bacilli of this class in the throats of a large percentage of persons in normal health has been demonstrated by the work of many observers in widely separated localities. It is, however, especially noteworthy that strains isolated from "influenzal meningitis"—which has never been proved to have any connection with influenza—manifest a distinctly greater immunologic (agglutinative) resemblance than do strains from influenzal pneumonia lesions or other sources. Rivers⁸ has pointed out that a very large proportion of the meningeal strains of the Pfeiffer bacillus produce indol, thus tending toward a cultural as well as an immunologic uniformity. It is tempting to conclude, therefore, that this group of hemophilic bacilli, in general of low invasive properties, may not only possess the capacity under appropriate conditions of developing varieties with somewhat exalted pathogenic powers, but that such a variety as that exemplified in the meningeal strains is actively in process of formation. The fixing of such a pathogenic type is a process accompanied by a stabilization of cultural and immunologic characters. With this view of the significance of uniform agglutination in the life of a bacterial species as the fixing of a biologic quality in connection with the acquisition of power to invade the animal body, the biologic position of these hemophilic bacilli becomes relatively clear. The failure of ourselves and of others to find consistent immunologic reactions among the strains isolated from epidemic influenza is a strong argument against the assumption of a primary causal relation between influenza and the Pfeiffer bacillus.

⁸ Jour. Am. Med. Assn., 1920, 75, p. 1495.

SUMMARY AND CONCLUSIONS

The particular strain of Pfeiffer bacillus used to produce an immune serum is usually agglutinated by the homologous serum more rapidly, more constantly and in higher dilution than any heterologous strain.

Absorption tests throw little if any more light on the true biologic relationship of the different strains in this group than does direct agglutination.

As a rule, each strain of Pfeiffer bacillus possesses a serologic individuality.

Occasionally strains from independent sources exhibit a serologic identity. This is not common.

As many as three serologic races may be present at the same time in the throat of one patient.

There is no correlation between indol-producing powers and agglutinative affinities, except possibly in the strains isolated from meningitis.

The lack of any definite serologic grouping among the strains of Pfeiffer bacilli is an indication that a distinct invasive type, or types, has not become fixed, and is an argument against regarding any member of this group as the primary causal agent in epidemic influenza. Possibly a race of "influenza meningitis" bacilli is in process of evolution.

FACTORS CONTROLLING THE INTESTINAL BACTERIA

THE EFFECT OF ACUTE OBSTRUCTION AND STASIS ON BACTERIAL TYPES

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The demonstration of the rôle played by intestinal bacteria in the toxemia of intestinal obstruction as developed by the authors¹ and the great probability of the importance of these organisms in other pathologic conditions makes it of considerable practical as well as theoretical importance to determine all of the factors which influence the types of intestinal flora. Kendall's² recent researches have indicated brilliant therapeutic possibilities in specific infections of the gastrointestinal tract which depend on the adequate control of either the types of intestinal bacteria or of their metabolic processes. The work of Kendall,² Hull and Rettger,³ Torrey,⁴ Cannon,⁵ Rettger and Cheplin⁶ and others has demonstrated that the types of intestinal bacteria depend to a great extent on the character of the diet. A diet rich in protein of animal origin brings about an intestinal flora dominated by proteolytic putrefactive organisms, while a carbohydrate diet, more particularly one containing definite amounts of either lactose or dextrin, produces a complete change in the intestinal bacteria so that the predominant organisms are fermentative or aciduric. Apparently many recent workers have lost sight of the more significant fact pointed out by Kendall that not only is a change in the types of bacteria effected by diet but also a change in their metabolism as determined by an analysis of the end products of their activity, in the one case when they must obtain their energy solely from protein and in the other when a readily utilizable carbohydrate is present.

Received for publication April 12, 1922.

¹ Dragstedt, L. R.; Moorhead, J. J., and Burcky, F. W.: Jour. Exper. Med., 1917, 25, p. 421. Dragstedt, L. R.; Dragstedt, C. A.; McClintock, J. T., and Chase, C. S.: Ibid., 1919, 30, p. 109. Dragstedt, C. A.; Dragstedt, L. R., and Chase, C. S.: Am. Jour. Physiol., 1918, 46, p. 366. Dragstedt, C. A., and Moorhead, J. J.: Jour. Exper. Med., 1918, 27, p. 359.

² Am. Jour. Med. Sc., 1918, 156, p. 157.

³ Jour. Bacteriol., 1917, 2, p. 47.

⁴ Jour. Med. Res., 1919, 39, p. 415.

⁵ Jour. Infect. Dis., 1920, 27, p. 139 and 1921, 29, p. 369.

⁶ Transformation of the Intestinal Flora, 1921.

It is the general view that the addition of lactose or dextrin to the diet brings about the change in the intestinal types because these carbohydrates, unlike others, are slowly absorbed and hence reach the region of maximum bacterial activity. Under these conditions it would seem that the suppression of proteolytic organisms, which grow best in a slightly alkaline medium, would be due to the accumulation of acid metabolites and the consequent change in the reaction of the medium. The aciduric bacteria, on the other hand, apparently prefer a slightly acid medium, and so would find conditions for their growth more favorable. Kendall maintains that when even small amounts of carbohydrates are present the proteolytic organisms obtain their energy from this source, and their metabolites are now acid in nature. These observations would suggest that with carbohydrate feeding both the aciduric and proteolytic organisms combine to form a medium favorable for the former and unfavorable for the latter. This interpretation is in accord with the well-known observation that the feces of the breast-fed infant are acid in reaction whereas those of an adult on a high animal protein diet are alkaline, and coincides with the facts in the natural souring of milk and the ripening of silage, where the evidence is convincing that the first stage is carried on by members of the colon-aerogenes group, and as the acidity increases these are outnumbered by streptococci and these in turn by lactobacilli.

Rettger and Cheplin, however, have found no relation between acidity of the feces and the presence of *B. acidophilus*, and they interpret the predominance of *B. acidophilus* as being the result of optimum cultural and environmental conditions for the development of members of the aciduric group. Whatever may be the exact factor in determining the predominance of types, it is apparent that diet is not the sole factor, as the bacteria are subjected to all the variations in functions of the intestinal tract, namely, motility, secretions, and degree of absorption.

EFFECT OF OBSTRUCTION ON BACTERIAL TYPES

We have previously reported experiments in lower animals in which the evidence seemed to prove that the toxemia of acute intestinal obstruction is directly dependent on the nature of the intestinal flora. The accompanying table includes the results of these experiments together with those of 2 more groups (groups 4 and 5) studied later. White rats were used and were divided into 2 groups, one being fed a meat diet and one a meat and lactose diet. Under these conditions

the undigested residues were more nearly comparable than in the preceding experiments. The diets were given for one week, and at the end of this time fresh feces of the animals were plated, and the results expressed in terms of the proportion of *B. coli* to *B. acidophilus* as the C-A ratio (Cannon⁵.) A complete obstruction in the lower colon was then produced by ligation with tapes. The diets were continued after the operation. All of the animals in which the obstruction remained complete died within 11 days and at necropsy an examination was made of the intestinal flora above the obstruction. The results are shown in the table.

It will be seen that the animals whose intestinal flora was aciduric survive the obstruction a little longer than those whose flora was proteolytic. There was, however, a much more marked difference in the postoperative behavior of these two groups. The meat eaters were listless

TABLE 1
RESULTS OF NECROPSY EXAMINATION

Groups	No. of Rats	Diet	C-A Ratio (Average)	Days Survived Obstruction	C-A Ratio After Death
1	3	Stock diet: oats, cabbage, carrots..	3 : 97	6½	90-10
2	15	Meat diet: ground beef, cabbage, carrots.....	87 : 13	7½	99 : 1
3	12	Lactose diet: whole milk, whole wheat bread, lactose, cabbage....	2 : 98	9½	97 : 3
4	4	Meat diet: ground beef.....	99 : 1	6	99 : 1
5	4	Lactose diet: ground beef 7 parts, lactose 3 parts.....	1 : 99	10½	98 : 2

from the first and refused their food. The lactose eaters, on the other hand, were alert and always eager for food. This is confirmatory of the conclusions of two of us in regard to the nature of the toxic substances in acute intestinal obstruction, namely, that they are produced by the activity of proteolytic intestinal organisms. The postoperative behavior of the meat eaters is also very similar to that of the monkey studied by Herter and Kendall⁷ which was sleepy and stupid when being fed a diet encouraging the growth of proteolytic organisms, but became alert when carbohydrates were added to the diet. More recently Underhill and Simpson⁸ have observed similar effects in a dog fed the two types of diet. The striking thing about these experiments, however, is that irrespective of the character of the diet, i. e., whether lactose is given or not, a complete stasis of the intestinal content results in a proteolytic intestinal flora.

⁷ Jour. Biol. Chem., 1908, 7, p. 203.

⁸ Ibid., 1920, 44, p. 90.

The results obtained in rats were entirely confirmed in dogs. Young animals were selected for this work and, confirming the observations of Torrey, it was found that diets of bread, milk, and lactose, or boiled rice, beef heart, and lactose in definite amounts would produce in these animals an aciduric intestinal flora. On the other hand, a diet of meat alone or of meat and boiled rice would develop a proteolytic flora. A complete obstruction was then produced in the transverse colon by section and closure of the divided ends. Following the operation the animals were kept on their respective diets. All of them died in the course of 2 weeks, those fed on the lactose surviving 2 or 3 days longer on the average than the meat eaters. At necropsy in all cases there was found above the site of obstruction a foul smelling, dark gray fluid, and examination revealed a great predominance of gram-negative proteolytic organisms.

BACTERIAL TYPES IN CLOSED INTESTINAL LOOPS

It has been demonstrated¹ that when closed isolated intestinal segments are made in dogs, the animal usually dies in 4 to 5 days of an acute toxemia developing from the isolated loop. The responsible toxic substances in the loop contents belong to the class of amines, and the rapid distention of the loop by the enclosed secretions so injures the intestinal wall that they are rapidly absorbed unchanged into the blood stream. Bacteriologic examination of the loop fluid from dogs which have previous to the operation been fed on the regular stock diet, shows a great preponderance of gram-negative organisms. The same is true of animals that have been fed meat previous to the production of the closed loop.

In these experiments isolated segments of the small intestine of dogs were made by cutting the intestine across in two places approximately 12 inches apart and then reestablishing the continuity of the alimentary tract by anastomosing the proximal and distal ends of the cut intestine. The isolated segments with intact mesentery were then closed at both ends and returned to the abdominal cavity. The operations were performed with aseptic precautions. Previous to the operation the dogs were fed either on a diet of bread, milk, and lactose, or boiled rice, beef heart, and lactose, and in each case the fecal flora was found to be strongly aciduric. At operation smears from the jejunal content revealed gram-positive organisms chiefly. These animals did not survive the production of a closed intestinal loop any longer than dogs fed on a meat diet. In every case at death the bacteria

in the closed loops were almost entirely gram-negative in type. This point was noted by Reynolds and McClintock.⁹ The loop fluid was alkaline to litmus.

Isolated closed intestinal loops previously washed with ether and sterile water usually show in 4 or 5 days an accumulation of great numbers of gram-negative organisms. These apparently live in the intestinal secretions, and the medium does not inhibit their growth. If isolated intestinal loops are washed with a saturated solution of tannic acid previous to closure, their distention and the consequent production of a toxemia is prevented. Animals may survive indefinitely with such closed intestinal segments. The loop contents examined after from several months to a year still show great numbers of viable gram-negative organisms. Apparently the succus entericus is not sufficiently bactericidal to suppress the growth of these organisms and is not comparable to the fluid in the free abdominal cavity in that respect. It is probable that the gram-positive bacteria disappear from these loops for the same reasons that they disappear from the normal intestine in starvation.

EFFECT OF STASIS ON BACTERIAL TYPES

An attempt to secure a slowing of the intestinal current without complete obstruction was not very successful. The best of the methods used was the isolation and inversion of an intestinal segment in the manner described by Mall.¹⁰ In 2 dogs an aciduric intestinal flora was produced by a diet of bread, milk, and lactose. Isolated segments of the intestine 12 inches in length were produced and then stitched back into place in the inverted position. It was assumed in accordance with the usual view of intestinal peristalsis that when the intestinal content would encounter this reversed segment a stasis would result unless the material could be mechanically squeezed through. Our results corroborated the findings of Beer and Eggers¹¹ and McClure and Derge¹² rather than those of Mall. There was evidence of an obstruction, with vomiting and loss of appetite lasting for a week or two, but thereafter the animal was active, had a good appetite and regular bowel movements. During the period of obstructive symptoms the fecal material was scant, and there was a tendency for the gram-

⁹ Personal communication.

¹⁰ Johns Hopkins Hospital Reports, 1896, 1, p. 93.

¹¹ Annals of Surgery, 1907, 46, p. 576.

¹² Bull. Johns Hopkins Hospital, 1907, 18, p. 472.

positive organisms to be replaced by gram-negative bacteria. When the obstructive symptoms disappeared and the bowel movements became regular the aciduric bacteria once more gained the ascendancy. The significance of this experiment is lessened because during the period of obstructive symptoms it was difficult to get the animals to retain the food.

DISCUSSION

Evidence is presented which indicates that other factors than the character of the diet are concerned in determining the type of intestinal flora. Either a complete obstruction or a stasis in the passage of the intestinal content results in a proteolytic flora irrespective of the character of the diet. It is probable, however, that the mechanism of bacterial control is identical in both cases and is dependent on the presence of utilizable carbohydrate in all parts of the small intestine and colon. In conditions of stasis or obstruction even such carbohydrates as dextrin and lactose are probably completely absorbed in the upper part of the intestine and so cannot affect bacterial growth lower down.

The experiments with the closed intestinal loops demonstrate that the intestinal juice is not markedly bactericidal, at least so far as the proteolytic group of organisms is concerned. The disappearance of the gram-positive aciduric bacteria is probably due to the fact that carbohydrates are absent from such loops and that the intestinal juice is quite markedly alkaline in reaction.

CONCLUSIONS

Intestinal stasis or complete obstruction leads to the development of a proteolytic intestinal flora irrespective of the character of the diet.

The intestinal secretions do not suppress the growth of gram-negative bacteria in closed intestinal loops.

The disappearance of gram-positive aciduric organisms from closed intestinal loops is probably due to the absence of utilizable carbohydrate and to the alkaline reaction of the medium.

OBSERVATIONS IN NONSPECIFIC IMMUNITY

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In analyzing the data collected in the medical examinations of students entering the University of Wisconsin during a period of 4 years, Van Valzah,¹ in 1914, reported that in this group of over 5,000 persons, the women gave a previous history of considerably greater percentage morbidity than the men in all the more common communicable diseases. The figures are given in table 1.

"In order to understand the more frequent occurrence of these diseases in the women, a study was made of the home environment of the various students and it was found that a large majority of the women came from towns and cities while a large proportion of the male enrollment came from rural districts." That the previous environment was an important factor in the striking difference in incidence of disease in the two sexes was further emphasized by figures obtained from observing the students during their 4 years of college life. The proportion of men to women in the University was about $2\frac{1}{2}$ to 1 whereas the morbidity relation in these diseases during University life was much greater than this, showing a ratio of 7 for the men to 1 for the women, a reversal of the figures for precollege life. The influence of previous city life is still further shown by the high incidence of communicable diseases among the "short course" agricultural students. This group of students, practically all of whom come from rural districts, made up at that time about 10% of the total student body and yet they showed a 31% incidence from these diseases.

Year after year, the figures in the university tell the same story. One year it is measles, the next mumps and the next possibly a streptococcus epidemic, but always if an outbreak occurs, the incidence is higher among the short course students than in the rest of the student body. Not only is the incidence greater among the men from strictly rural communities, but also when they are ill their illness is of greater severity, as a rule, than is that of the regular students. In epidemics when the temperature averages 102 F. the short course students commonly have a temperature of 103-104 F., and present a more severe

Received for publication, May 4, 1922.

¹ Wis. Med. Jour., 1915, 14. p. 1.

clinical picture.² During the influenza epidemic of 1918,³ for example, the death rate for this group was 3.21% while for the rest of the student body, including the S. A. T. C., the death rate was 0.82%.

This same point has been brought out on a much larger statistical basis by Love and Davenport⁴ and by Vaughan and Palmer⁵ as a result of their findings in the army cantonments during the recent war. These observers have noted that not only was the morbidity and mortality from the common infectious diseases greater among the men from the sparsely settled rural communities, but this was also true for the less common diseases, such as epidemic meningitis and pneumonia. Except for some slight irregularities, the admission rate for measles, mumps, scarlet fever, primary lobar pneumonia and epidemic menin-

TABLE 1
INCIDENCE OF COMMUNICABLE DISEASES IN STUDENTS BEFORE ENROLLMENT IN
UNIVERSITY

Number of Students	Measles, Percentage	Whooping Cough, Percentage	Mumps, Percentage	Chicken Pox, Percentage
Men.....3,955	79	46	60	40
Women.....1,780	92	67	65	63
Total.....5,735				

gitis was in approximately inverse ratio to the density of the population in the regions from which the troops came. This is shown most strikingly, for example, in the number of cases of measles occurring in 1917 at Camp Wheeler, Ga., and at Camp Wadsworth, S. C. These camps were only 100 miles apart and located in the same general type of country. Measles was present at both camps, yet at Camp Wheeler the mean annual admission rate was 500 per 1,000 while at Camp Wadsworth it was about one in 1,000. An examination as to the homes of these troops showed that the soldiers at Camp Wheeler were recently recruited National Guard troops from Virginia, Alabama and Florida, whereas the men at Camp Wadsworth were seasoned National Guard troops from New York City and its vicinity.

It would seem to be an epidemiologic fact, therefore, that when persons from rural communities are brought suddenly into close associa-

² Middleton, W. S., and Van Valzah, R.: Personal communication.

³ Van Valzah, R.: Influenza Among University Students, Report to Board of Regents, University of Wisconsin, 1918.

⁴ Arch. Int. Med., 1919, 24, p. 129.

⁵ Military Surgeon, 1920, 46, p. 1.

tion with many persons, they exhibit a lower resistance to the ordinary communicable diseases than do their city bred brethren.

There are several obvious explanations for this fact. Doubtless the most important factor, in the greater resistance of the "city dwellers," is, as Van Valzah pointed out, an actual specific immunity acquired during the course of recovery from recognized cases of measles, whooping cough, chicken pox, etc. The incidence of such acute diseases is in most parts of the United States higher among city children⁴ than among the children in the farming communities. In table 2, are figures obtained from Dr. I. F. Thompson of the Wisconsin State Board of Health, which show that in two of the less common communicable diseases also, namely, typhoid fever and pneumonia, the mortality, and

TABLE 2

DEATH RATE IN WISCONSIN RURAL AND URBAN COMMUNITIES, URBAN BEING INTERPRETED AS POPULATIONS OF 2,500 OR MORE

	1910		1911		1912		1913		1914	
	Urban	Rural	Urban	Rural	Urban	Rural	Urban	Rural	Urban	Rural
Typhoid fever rates per 10,000 population.....	3.5	2.0	1.8	0.9	1.8	0.8	2.4	0.6	0.9	0.2
Pneumonia rates per 10,000 population.....	4.1	5.3	15.9	7.6	12.4	5.7	12.6	5.9	Not obtained	Not obtained

undoubtedly the morbidity as well, has been lower in the rural communities of Wisconsin than in those of over 2,500 inhabitants, considered as urban. It is recognized that in the case of typhoid fever, this relation between urban and rural districts would not hold throughout the country, as this disease is one which in many parts shows a higher incidence in the rural districts. In Wisconsin, the high rate for urban communities has been largely due to the conditions prevailing in the cities situated on Lake Michigan, where that body of water is used both as a source of water supply and as a means of sewage disposal.

One of the difficulties in obtaining adequate figures relative to morbidity and mortality is that the U. S. Census Bureau has used 10,000 as the arbitrary division between rural and urban communities, and this point of division has been generally adopted. We feel that this is not a suitable dividing line as many communities with a population of 4,000-5,000 have the disadvantages of the close associations of city life without the advantages, such as an adequately supervised public water supply, sewage disposal, etc.

Aside from the greater incidence of recognized cases in city communities, there is also the undoubted occurrence of unrecognized mild attacks. The possibility of a higher incidence of such abortive attacks is obviously greater in the more closely packed communities than in rural districts. This also would tend to produce specific immunity to the common communicable diseases.

Zingher⁶ reports an interesting instance of specific immunity in a densely populated urban district as contrasted with a less densely populated area. He states that the number of positive Schick tests was definitely greater among the children from the homes of the more well-to-do of New York than among the children of the community in general and that the lowest number of positives was found among the children of the slums. Presumably, a comparison of city and country communities would show an even greater contrast.

Furthermore, the higher mortality rate in densely populated city districts may result in a certain amount of natural selection of those best fitted to survive in a rich and varied bacterial environment, the weaker members failing to reach maturity. This may well be a factor in diseases in which the mortality is high, but it is hard to see how it would play any considerable part in a disease like mumps, for example, in which fatalities among children are practically nil.

The factor of specific immunity, then, is admittedly the most important point in rendering adult city dwellers more resistant than their rural brothers when brought in contact with infectious agents. Is it not possible, however, due to the greater interchange of organisms and the higher incidence in general of communicable diseases in our cities, that a nonspecific immunity of some degree may be developed? Is there a nonspecific immunity entirely apart from the well recognized "group reactions?" Through repeated slight injuries to the antibody producing cells, should we not expect on a pathologic basis a hyperplasia of these tissues, an actual extension of the lymphoid tissue or the bone marrow for example? Such a training of the antibody-producing mechanism should result either in a more rapid response in the production of antibodies or in the production of more antibodies as the result of a given stimulus.

The greatest emphasis on nonspecific factors in resistance has come largely from clinical medicine. Nonspecific bacterial vaccines, milk, normal serum, protein-split products, enzymes, tissue extracts as well as colloidal metals have all been employed in recent years in the treatment of almost every com-

⁶ Jour. Amer. Med. Assn., 1921, 77, p. 835.

municable disease but more especially in the treatment of different types of arthritis and typhoid fever. Auld in Great Britain, Chantemesse in France, R. Kraus and Ludke in Germany, and J. L. Miller in this country are among the leading exponents of this type of therapy and have all reported numerous successful results in its application.

From the laboratory side, the finding by Vaughan and Wheeler⁷ of apparently the same toxic elements by the hydrolytic cleavage of either pathogenic or nonpathogenic bacteria or from altogether nonbacterial sources is of great importance in this connection. V. C. Vaughan, Jr.,⁸ succeeded in building up a nonspecific resistance in experimental animals by several injections of this toxic element. These animals rarely, however, withstood more than a single fatal dose whereas with the nontoxic specific fraction, an active immunity could be induced so that such animals would withstand many fatal doses. Animals immunized with the nonspecific elements did not show any demonstrable immune bodies.

Bacteriologists have always delighted in correlating this work of Vaughan with the splitting of protein through the use of alexin and specific antibody (Friedberger, Friedman, etc.) resulting in the production of nonspecific toxic substances commonly known as anaphylatoxins. These two lines of work have furnished support to the contention that difference in clinical picture is due largely to such factors as location of the lesion, invasive properties of the bacteria, etc., and that specific endotoxins do not exist.

Jobling and Petersen,⁹ approaching the problem from another angle, have, in a series of papers, demonstrated the mobilization of nonspecific proteases and lipases in the blood as important factors in resistance.

Hektoen,¹⁰ Bieling,¹¹ Pinner and Ivancevic,¹² and Hermann¹³ have all shown that the injection of a second and different protein will call forth an increased amount of antibodies against the antigen first used in immunization.

We have approached the subject from still another point of view, that of the striking differences in morbidity observed year after year among the various groups of students at the University of Wisconsin. The following experiments were planned, therefore, to reproduce, if possible, some of the conditions prevailing among persons living in urban and rural surroundings with the hope of obtaining some suggestions in answer to the questions stated in the foregoing.

Twelve rabbits were selected, weighed, bled—3 or 4 c.c. from each animal—for a normal serum sample and then placed in individual cages. They were divided into 2 groups, the first (1-6) to be made into "city dwellers" by a series of vaccinations, and the second (7-12) to remain under the same conditions as to care and feeding, but to receive no treatment, and therefore to be called "country dwellers." We

⁷ Protein Split Products, 1913.

⁸ Jour. Med. Research, 1905, 14, p. 67.

⁹ Arch. Int. Med., 1917, 19, p. 1042.

¹⁰ Jour. Infect. Dis., 1917, 21, p. 219.

¹¹ Ztschr. f. Immunitätsf., 1919, 33, p. 246.

¹² Ibid., 1920, 30, p. 542.

¹³ Jour. Infect. Dis., 1918, 23, p. 457.

planned then to inoculate both groups of animals with an antigen differing widely from the vaccines used for the city dwellers and to titrate the serum of each animal for antibodies developed against this second antigen over a period of 3 weeks.



Chart 1.—Agglutination curves of vaccinated and unvaccinated animals, intravenous series.

Vaccines sufficient for the whole series of injections were made with 5 organisms, 2 different strains of *Streptococcus viridans*, 1 hemolytic streptococcus, a *Staphylococcus aureus* and a pneumococcus, type undetermined. All of these organisms had been recently isolated.

These particular species of bacteria were chosen because of their prevalence in many infections in the human upper respiratory tract. Salt solution suspensions were made from 24-hour cultures grown on Löffler's blood serum and then sealed in glass and heated to 63-65 C. for 50 minutes.

Subsequent plantings showed that all vaccines were sterile except that a few colonies grew from one of the viridans vaccines. All suspensions were made up to have an opacity equal to that of an equal thickness of a 0.3% lecithin solution.

The first group of animals (rabbits 1-6) was injected intravenously at 5 day intervals with 1 c c of each vaccine separately as follows:

- 17-3-21 *Staphylococcus aureus* (Stock culture)
- 22-3-21 *Streptococcus hemolyticus* (Sprague)
- 26-3-21 *Streptococcus viridans* (Robertson)
- 1-4-21 *Pneumococcus* (Young)
- 6-4-21 *Streptococcus viridans* (Clark)

A rest of eleven days was then allowed during which the M. L. D. of *B. typhosus* was determined. This organism was chosen because of its marked differences from the organisms used for the preliminary vaccinations, so that no possibility of group reactions, as that term is ordinarily used, could enter into the problem. The organisms used for vaccination were all cocci, the secondary antigen was a bacillus. The cocci were all gram-positive, *B. typhosus* is gram-negative, with all the essential differences in protein that that staining reaction implies. The cocci used all stimulate the bone marrow to produce a polymorphonuclear leukocytosis, whereas the typhoid bacillus produces a lymphotoxin.

The *B. typhosus* used was an old stock culture and relatively non-virulent. As a result of 2 series of injections, the M. L. D. was found to be approximately three 24-hour slant agar cultures. Accordingly, a dosage of 2 cultures was determined on.

On the twelfth day after the last injection of cocci, therefore, both the vaccinated and the unvaccinated groups of animals were injected intravenously with *B. typhosus*. In order to prolong the injuring effect, the dose was divided and one culture of the living organisms given on that day, and one on the succeeding day.

One of the "city dwellers" (rabbit 6) died during the night following the first injection of *B. typhosus* and one (No. 4) was eliminated from the series because of an extensive subcutaneous staphylococcus abscess which was causing considerable loss of weight. Within 15 hours

after the second injection of *B. typhosus* rabbits 7, 8, 9, 11 and 12, all "country dwellers," were dead, whereas the 4 city dwellers were well and happy. At necropsy all of these animals showed marked injection of the intestinal vessels with no other positive findings, save positive blood cultures in 3 instances, and parenchymatous changes in the viscera.

This surprising result made it necessary to obtain more "country dwellers," if possible, in order to carry out the original plan. Also we endeavored to discover whether the divided dose was to any degree responsible for the apparent sudden increase in the virulence of the *B. typhosus*, or whether it was a decrease in the normal resistance of

TABLE 3
AGGLUTINATION—FIRST SERIES

Days of Bleeding	"City Dwellers"				"Country Dwellers"	
	1	2	3	5	10	18
Normal: before treatment	—	—	—	—	1:5+	1:5+
1 day after injection of <i>B. typhosus</i>	1:5+	1:5+	—	1:10+	—	—
3 days after injection.....	1:160+	1:80+	1:80+	1:160+	1:40+	1:40+
6 days after injection.....	1:2560+	1:320+	1:1280+	1:1280+	1:640+	1:320+
9 days after injection.....	1:640+	1:320+	1:1280+	1:2560+	1:320+	1:640+
12 days after injection.....	1:640+	1:80+	1:640+	1:2560+	1:160+	1:160+
17 days after injection.....	1:160+	1:160+	1:640+	1:320+	1:80+	1:80+
22 days after injection.....	1:80+	1:80+	1:320+	1:320+	1:80+	1:20+

+ signifies the highest dilution at which definite macroscopic agglutination was obtained. The Stern and Korte dilution method was used.

— signifies no macroscopic agglutination in the highest concentration used (1:5).

our animals. Accordingly, 6 more normal animals (13-18) were injected with *B. typhosus*, one pair with two 24-hour slant cultures in one dose each, another pair with a divided dose, one culture on the first day and a quarter of a culture on the second; and a third pair with a divided dose of 2 cultures each as in the case of the original series. The first pair receiving the undivided dose died promptly and one each of the other 2 pairs died within 24 hours. Since the surviving member of the third pair had received the same treatment as the original "country dwellers" group, this animal was added to that series as No. 18. The matter of the divided dose was not completely settled, but the evidence pointed to a sudden increase in the virulence of the organism used rather than to the divided dose as the important factor.

The 6 rabbits (4 "city dwellers" and 2 "country dwellers") were bled from the ear veins on the 1st, 3rd, 6th, 9th, 12th, 17th and 22nd day after the injection of *B. typhosus*. The ears were shaved and

treated with tincture of iodine and then alcohol in the usual manner. About 50 drops of blood were collected each time and the serum obtained was preserved in sealed glass tubes in the icebox until all were ready for titration.

The typhoid organism used for agglutination was the same strain as that used in the inoculations. It was grown in large flask cultures on agar and, after 48 hours' incubation, the organisms were suspended in isotonic salt solution and shaken in a mechanical shaker for 8 hours. An excellent homogeneous suspension was thus obtained and the macroscopic method of agglutination was employed with dilutions from 1:5 up to 1:5120. The same suspension was used for all the titrations. The tubes were incubated for one hour, allowed to stand at room temperature over night, and read the following morning. The serums from each of the "country dwellers" reached the highest point at 1:640, while 2 of the city dwellers reached 1:2560, one reached 1280, and one 320. The composite curves of the 2 groups are shown in chart 1. The individual figures are given in table 3.

DISCUSSION

Obviously, the striking result in these experiments is the difference in mortality between the "city dwellers" and the "country dwellers" when injected with the same dose of an organism, unrelated to the bacteria previously used for vaccination. Under this condition, the death rate among the "city dwellers" was 20%, whereas the loss among the country dwellers was 83-1/3%. As a partial explanation for this difference in death rate, we find that agglutinins in the group previously vaccinated with cocci attained an average level of 1:1360, whereas the average level of 2 unvaccinated animals was 1:480. This difference in the agglutinin curve in such a small series of animals would deserve less consideration did it not harmonize with the outstanding difference in the death rate. Aside from furnishing an antibody explanation of the mortality, this correlation between agglutinins and death rate seems to us an indication that agglutinins do not deserve the poor reputation they have been acquiring of late as indicators of immunity.

In repeating these experiments, an attempt was made to approximate more closely the conditions prevailing in the human body by introducing the primary antigen in small daily doses and the secondary antigen by the intratracheal route instead of intravenously. Two sets of rabbits were used, 5 in each group. Animals 1-5 were inoculated

intravenously daily for 21 days with a mixed vaccine made up of *Staphylococcus aureus*, *Staphylococcus albus*, *Streptococcus buccalis* and 2 strains of pneumococci, type undetermined. During the last 2 weeks of this period the vaccines used were attenuated and not completely killed, a slight growth being obtained from a heavy seeding of the control tubes. An amount equivalent to $\frac{1}{10}$ th of a slant culture was injected in each instance.

After the period of preliminary vaccination, all the animals were bled from the ear vein about 50 drops each and were injected intratracheally by passing a somewhat blunted needle directly through

TABLE 4
AGGLUTINATIONS—SECOND SERIES

Days of Bleeding	"City Dwellers"			"Country Dwellers"			
	1	3	5	6	7	9	10
After preliminary vaccination; before <i>B. typhosus</i> injection.....	1:10+	1:10+	1:10+	—	—	—	—
2 days after injection of <i>B. typhosus</i>	1:40+	1:40+	1:10+	—	1:20+	Serum contaminated	1:10+
4 days after injection.....	1:160+	1:80+	1:80+	1:40+	1:80+	1:40+	1:80+
6 days after injection.....	1:160+	1:80+	1:160+	1:80+	1:160+	1:40+	1:80+
10 days after injection.....	1:160+	1:160+	1:320+	1:160+	1:40+	1:160+
11 days after injection.....	1:320+	1:160+	1:160+	1:160+	1:160+	1:40+	1:80+
13 days after injection.....	1:640+	1:320+	1:80+	1:160+	1:320+	1:70+	1:80+
18 days after injection.....	1:40+	1:80+	1:80+	1:40+	1:320+	1:80+	1:160+
20 days after injection.....	1:40+	1:40+	1:40+	1:40+	1:160+	1:80+	1:160+

the skin and tracheal wall during ether anesthesia. Eight slant agar cultures of *B. typhosus*, a sublethal dose, were then forced deeply into the lungs by using a large syringe.

After this injection, animals 1-4 did not appear sick whereas the unvaccinated group showed loss of appetite and appeared generally toxic for about 48 hours. One of the animals, No. 2, died as the result of a mixed infection on the day following the typhosus injection, later No. 4 broke his back during bleeding and No. 8 developed severe ear lesions early in the experiment and was discarded. The remaining animals were bled on the 2nd, 4th, 6th, 10th, 11th, 13th, 18th and 20th days following the injection of *B. typhosus* (table 4). In this series, we again note a distinct difference in the reaction of the 2 groups, the group previously vaccinated with Gram-positive cocci responding with more than twice the concentration of agglutinins against the secondary antigen, *B. typhosus*, than did the normal unvaccinated

animals. Neither group of animals in this series produced antibodies to as great a degree as did the intravenous series, but that is to be expected because of the difference in route of injection (Clark and Murphy ¹⁴).

CONCLUSIONS

By the intravenous injections of repeated doses of gram-positive cocci, rabbits are rendered more resistant to the injection of a totally unrelated organism, *B. typhosus*.

This type of nonspecific vaccination causes the rabbits to respond, when subsequently inoculated with *B. typhosus*, by building up a higher concentration of agglutinins against this unrelated antigen than do normal animals kept under the same living conditions.

Is it not probable that similar nonspecific immunity may be built up because of the rich and varied bacterial environment of our city life and may be in part responsible for the greater resistance of our city bred students as compared with those reared in rural isolation?

¹⁴ Jour. Infect. Dis., 1922, 31, p. 51.

COMPLEMENT FIXATION IN TYPHOID FEVER

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In the last few decades, with the steady advancement of our serologic and bacteriologic knowledge of typhoid fever, new and efficient means of diagnosing the disease have been put at our command. But of all such tests only those that combined simplicity with specificity survived. For these reasons the Gruber-Widal reaction was adopted by health centers throughout the civilized world as indispensable for the diagnosis of typhoid fever, although, as a reaction, it is not the earliest immune manifestation nor is its course uniform.

The field of complement-fixation tests for typhoid is one of the least explored. When one stops to think that typhoid fever was one of the first diseases in which the phenomenon of complement deviation reaction was demonstrated by Bordet and Gengou¹ in 1901, one wonders why so few have taken up the subject later.

The reports of recent investigators on complement fixation in typhoid are conflicting. Rodet and Faber² were unable to demonstrate complement fixing bodies in animals immunized against typhoid, while Kolmer,³ working similarly, obtained positive results. The complexity of reaction, the effect of heating on the immune bodies of serum and the indiscriminate choice of antigen are, no doubt, the main causes for such conflicting reports.

We have lately developed a simple differential quantitative technic for active serum complement fixation which has been applied successfully to the diagnosis of syphilis, gonorrhea and to a limited degree of tuberculosis.⁴ The successful application of the same technic to typhoid is described.

TECHNIC

Human serum is used while fresh; it should not be over 48 hours old. After keeping the serum together with the clot for 24 hours it is separated by centrifugalization and distributed equally in 6 pairs of small test tubes in doses of 0.01, 0.02, 0.03, 0.04, 0.05 and 0.10 cc,

Received for publication, May 8, 1922.

¹ Ann. de l'Inst. Pasteur, 1901, 3, p. 289.

² Phys. et Path. Gén., 1912, 14, p. 86.

³ Jour. Med. Research, 1912, 21, p. 373.

⁴ In press.

respectively. The left hand series of tubes receive 0.5 c c of salt solution each and serve as controls for the hemolytic value of the serum in question. The right hand tubes receive 0.5 c c of the bacillary antigen each. The tubes are placed at 37 C. for 30 minutes and then 0.5 c c of one half % suspension of sensitized sheep cells are added to all. Readings are taken in 15 to 30 minutes or as soon as the complementary value shows a unit of hemolysis in 0.02 or 0.03 c c of serum.

Certain serums are naturally deficient in hemolytic value. In such cases the serum in question is mixed with an equal amount of a typhoid-negative serum of high hemolytic value and the test carried on as usual. The ratio of the hemolytic values in the two series of tubes indicates the degree of specificity in terms of fixed complement units. The whole process does not take more than two hours from the time the

TABLE 1

TYPHOID COMPLEMENT-FIXATION READINGS OF SOME CASES OF TABLE 2 *

Case	Amount of Serum in C c						Specific Ratio	Plus Equivalent
	0.10	0.05	0.04	0.03	0.02	0.01		
15	C/C	C/3	C/3	C/3	C/3	1/3	0.060 : 0.015	++++
16	C/C	C/1	C/3	C/3	C/3	1/3	0.045 : 0.015	+++
19	C/C	C/3	C/3	C/3	2/3	3/3	0.050 : 0.025	++
26	C/C	C/C	C/1	C/2	C/3	2/3	0.020 : 0.020	+
27	C/C	C/C	C/C	C/C	1/2	2/3	Negative

* C indicates complete hemolysis; 3, complete inhibition of hemolysis. Numerals 1 and 2 indicate roughly $\frac{1}{2}$ and $\frac{2}{3}$ inhibition. Of the two readings under each amount of serum the first indicates the hemolytic and the second the specific value. For example, in case 16 the hemolytic unit is 0.015 c c and the specific 0.045 c c, the specific quotient being 0.045 : 0.015 equals 3 units of fixation, roughly ++++. In case 27 as the specific value does not exceed the hemolytic, the case is considered negative.

specimen of blood is received. Table 1 gives actual duplicates of the readings of a few of our cases as given in table 2.

The success of the test depends mainly on the proper choice of antigen, and it must be born in mind that organisms belonging to the same species may be differentiated immunologically. As a nucleus for our antigen we collect 8 or 10 typhoid vaccines from various sources. Then we test the maximum and minimum antigenic values of this mixture against 0.02 c c of typhoid immune rabbit serum, the amount of the latter depending on its potency. The antityphoid serum of New York Department of Health usually represents one unit of complement fixing value in 0.01 c c. The next step is to find the optimum antigenic value of the mixture by testing out its maximum nonspecific value against typhoid-negative human serums. For example, if we suppose the maximum antigenic dose, 0.50 c c, to be represented in a dilution of

TABLE 2

WEEKLY BACTERIOLOGIC AND SEROLOGIC RESULTS IN FIFTY CASES OF TYPHOID FEVER

Case		First			Second			Third			Fourth			Fifth			Clinical Notes
		BC	CF	AG	BC	CF	AG	BC	CF	AG	BC	CF	AG	BC	CF	AG	
1	R5	+	+	+	—	+	—	—	+	—	+	Moderately toxic
2	+	+	—	+	+	..	Regular course
3	..	+	+	—	+	..	+	+	..	Toxic
4	..	+	+	+	Toxic
5	..	+	+	..	+	+	—	..	+	+	Regular course
6	+	+	—	..	+	+	Moderately toxic
7	+	+	+	Died in coma on admission
8	—	+	—2	±	Regular course
9	R3	+	+	..	+	+	+	Died, Staph. aureus sepsis
10	R3	+	—	+	—2	±	—	—	+	—2	Moderately toxic
11	+	+	+	+	+	Regular course
12	..	+	+	—	Toxic
13	—	+	+	Regular course
14	R4	+	+	+	—	+	—2	Regular course
15	—	+	+	+	..	Regular course
16	R4	—	+	..	—	—	—	..	+	Regular course
17	+	+	+	+	Died, Staph. albus sepsis
18	—	+	+	Regular course
19	+	Died, internal hemorrhage
20	—	+	—	—	+	+	—	+	+	Moderately toxic
21	+	+	+	+	Regular course
22	—	+	Regular course
23	R4	—2	±	—2	+	Regular course
24	..	+	+	+	Moderately toxic
25	+	+	+	Regular course
26	..	+	+	+	+	Regular course
27	..	+	+	+	—	—	—	Regular course
28	..	+	+	+	+	Moderately toxic
29	—	+	—	Regular course
30	—	+	—	..	+	Regular course
31	—	+	+	—	Regular course
32	+	+	—	+	+	Regular course
33	—	—	+	+	Regular course
34	R3	—2	—	+	Moderately toxic
35	—	+	—2	Regular course
36	..	+	—	—2	Toxic
37	—	—	Regular course
38	+	+	Regular course
39	—	—2	—	Regular course
40	±	±	Moderately toxic
41	..	+	+	—4	+	+	—3	—	Moderately toxic
42	..	+	+	—	Moderately toxic
43	..	+	—	—2	—2	Moderately toxic
44	+	—	+	Regular course
45	+	+	Moderately toxic
46	R4	..	+	—	—	—2	—	—	..	—	Moderately toxic
47	+	—	..	+	—2	Moderately toxic
48	—	±	—	—	—	—	Moderately toxic
49	±	..	Moderately toxic
50	—	+	Toxic
Total +..		15	13	11	10	21	18	6	22	17	..	2	7	..	3	2	Blood culture average % 44+
Total —..		1	6	12	17	7	18	20	2	12	1	..	1	1	..	1	Complement fixation average % 80+
% +.....		94	68	50	40	75	50	23	92	60	..	100	88	..	100	70	Agglutinin reaction average % 55+

BC, CF, AG, indicate blood culture, complement fixation and agglutination, respectively. R indicates relapses and recrudescences, and the numerals that follow it immediately indicate the week during which they have occurred. The reactions and cultural findings are reported either as positive, + or negative, —. Each sign stands for a single test; consequently ± means a negative and positive finding. The numerals following the signs indicate the number of repeated tests during the weekly interval.

1:50 and the minimum in a dilution of 1:100, by testing out intermediate dilutions between these two values we finally reach the optimum dilution, the last in the descending scale, that will give a clear negative reading as in case 27, table 1.

An antigen prepared and standardized in this way keeps for months in the icebox and answers the average demands. In hospitals, however, where blood cultures are taken in all septic cases, one at times meets cases of typhoid fever with positive blood culture and negative complement fixation. By further enriching the antigen with such rare strains a very potent antigen may be developed capable of detecting at least 80% of cases in practically all stages of the disease, from the first to the fifth week.

TABLE 3
COMPARATIVE CLINICAL VALUE OF BLOOD CULTURE, COMPLEMENT FIXATION AND AGGLUTINATION IN THE DIAGNOSIS OF TYPHOID

Type of Reaction			Number of Cases	Percentage
Blood Culture	Complement Fixation	Agglutination		
+	+	+	12	30
—	+	+	9	22
—	+	—	7	18
+	—	—	6	15
+	—	—	3	8
—	—	+	2	5
—	—	+	1	2
Total.....	40	100
50	85	60	Average percentage per reaction	

This technic has been applied to 50 cases of typhoid and over 100 control cases. The complement fixations were controlled by blood cultures and agglutination tests. We were thus able to make a comparative study of the efficiency and specificity of complement fixation and the other two tests in the diagnosis of typhoid fever.

The technic used in agglutination was the dried blood, microscopic, loop method, with two dilutions of serum; 1:10 and 1:50. Complete agglutination with complete loss of motility in both dilutions is considered positive. On the other hand, complete agglutination in both with partial loss of motility either in one or both dilutions, and vice versa, is considered as doubtful. With clear negative controls, both types of reaction are highly specific, as the average percentage of non-specific agglutinations, considering even doubtful reactions as positive, was within 8 (table 4). For the blood cultures we used no special

mediums. About 10 cc of ordinary nutrient agar were inoculated with 2 cc of blood and 3 or 4 plates poured. Growth, if any, invariably appeared within 3 days; otherwise cultures were considered negative.

The weekly results in the 50 cases of typhoid fever are given in table 2. In table 3 we have separated the cases in which all 3 tests show the degree of superiority of each test in the clinical course of the disease. The resumé of table 2 incorporating also our control cases is given in table 4 and shows the percentage of specific and nonspecific reactions. Finally, in chart 1, we have tried to give a visual presentation of the weekly course of each reaction on a percentage basis.

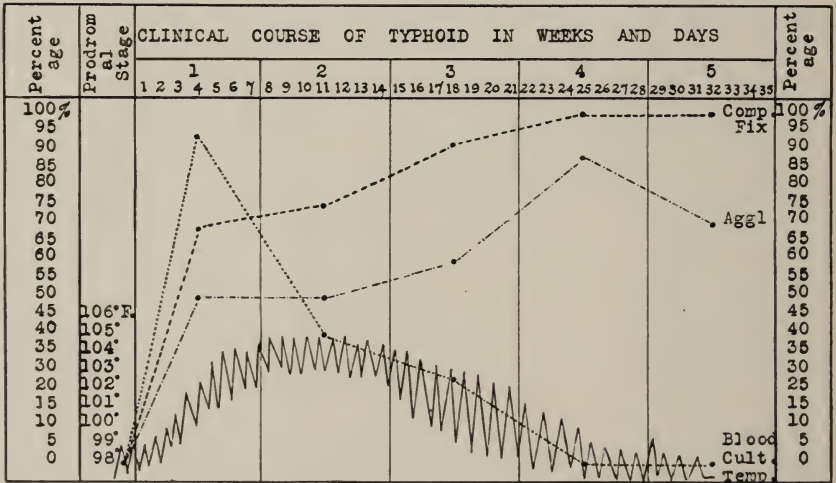


Chart 1.—The weekly course of blood culture and immune reactions in typhoid fever.

The tables and chart emphasize the significance of complement fixation as a diagnostic means of great efficiency in detecting typhoid fever. It is true that during the first week of the disease the blood culture is superior to the immunologic tests; but beginning with the second week, while the blood culture curve falls abruptly, the immune reaction curves rise steadily, the fixation curve always preceding. During the fourth week the agglutinin curve reaches its peak at 88%, thereafter showing a tendency to fall, while the complement fixing curve, from the fourth week and on, stands at 100%. What happens to it after the fifth week we are not in a position to say, as our investigations do not extend beyond the hospital stay of the patients.

As this study was undertaken to investigate primarily the value of complement fixation as a diagnostic means, the question arises whether by using special medium for blood culture and a more sensitive method for agglutination better results might not have been secured. Coleman and Buxton,⁵ with their special bile-broth medium for blood cultures in typhoid fever, obtained 69% positives. They also recapitulate the

TABLE 4

TOTAL CASE AND PERCENTAGE RECORDS OF BLOOD CULTURE, COMPLEMENT FIXATION AND AGGLUTINATION IN 50 TYPHOID AND 100 NONTYPHOID CASES INCLUDING NONSPECIFIC REACTIONS

Type of Disease	Type of Reaction	Blood Culture		Complement Fixation		Agglutination	
		Number of Cases	Percentage	Number of Cases	Percentage	Number of Cases	Percentage
Typhoid.....	Positive	31	45	61	80	55	55
Typhoid.....	Negative	40	55	15	20	44	45
Not typhoid.....	Positive	4	2*	16	8*
Not typhoid.....	Negative	29	100	120	98	85	92
Total.....		100	...	200	...	200	

* The percentages of nonspecific reactions were computed on the total number of tests, including typhoid as well as nontyphoid cases.

TABLE 5

PERCENTAGE COMPARATIVE TABLE OF OUR WEEKLY POSITIVE RESULTS WITH THOSE OF OTHER INVESTIGATORS

Weeks	Blood Culture		Complement Fixation, Author's Percentage	Agglutination	
	Coleman and Buxton	Author's Percentage		Author's Percentage	Park and Williams
First.....	89	94	68	50	20
Second.....	73	40	75	50	60
Third.....	60	23	92	60	89
Fourth.....	35	0	100	88	90
Fifth.....	26	0	100	70	75
Average*.....	69	45	80	55	65 ?

* The average percentages were computed on the total number of tests.

blood culture work in typhoid up to 1917, including the results of 1,602 individual blood cultures by 33 European and American investigators, the average percentage of positives being 75%. The weekly findings of Coleman and Buxton, together with the weekly agglutination reports of Park and Williams,⁶ are compared with our findings in table 5.

In table 5, despite the minor variations in the weekly findings, the slope of the corresponding curves is practically the same as in chart 1.

⁵ Am. Jour. Med. Sc., 1907, 133, p. 896.

⁶ Path. Microorganisms, 1917, p. 345.

In blood cultures the peak is reached in the first week and in agglutinations during the fourth week, which means that blood culture is of minor value in the latter and agglutination in the early course of the disease.

The pathologic conditions giving rise to nonspecific complement fixation, with the exception of those in one case of typhus, clinically also could be differentiated from typhoid. On the other hand, nonspecific agglutination occurred almost 100% in acute infectious diseases such as typhus, malaria, miliary tuberculosis, acute mastoiditis, acute appendicitis and influenza, that could not be easily differentiated from typhoid without further clinical and laboratory data.

SUMMARY

In the course of typhoid infection, the formation of complement-fixing antibodies is one of the earliest and most constant immune manifestations.

The introduction of a differential quantitative, active serum technic for the complement-fixation test makes it possible to utilize this property for the diagnosis of typhoid fever, with satisfactory results.

In a limited number of typhoid cases the test has been repeated with inactive serum and found similarly satisfactory. It is recommended, however, that the active serum technic be used, as it saves much time in the routine work of the test.

THE CULTIVATION OF BACTERIUM ABORTUS BANG*

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Many methods have been described for the cultivation of *Bact. abortus* Bang. Following the original work with this organism by Bang and Stribolt in 1897, several investigators have described methods for the cultivation of this germ. The most frequently mentioned and used are those of Nowak, Holth, Priez and Fabyan. Recently Stafseth¹ and Huddleson² have described culture mediums and methods of growing *Bact. abortus* which differ from those already in use. The former recommends a medium prepared from liver and spleen. He states that "Strains of the abortion bacillus have been isolated more easily by the aid of these media." A glass jar from which the air was partially exhausted by a suction pump was used in which to grow the cultures. Huddleson emphasizes the importance of an increased carbon dioxide tension for growing *Bact. abortus* Bang. His conclusions are:

There is sufficient proof that:

- (1) The growth of *Bact. abortus* is not due to a reduced oxygen tension.
- (2) A carbon dioxide tension greater than that of the air governs and greatly facilitates the primary growth of *Bact. abortus*.
- (3) An atmosphere containing (by volume) 10 per cent. of CO₂ gas appears to produce the earliest and most luxuriant growth of *Bact. abortus*.

Huddleson recommends the use of a generator containing calcium carbonate to which hydrochloric acid is added as a source of the CO₂.

We have been working with *Bact. abortus* for many years and have experienced the same difficulty as other authors in isolating the organism. All the methods mentioned have been used with more or less success. The abortion germ usually grows with great difficulty in the cultures made from the original material as the stomach contents of an aborted fetus. After two or three transfers, the bacilli grow quite readily even under ordinary aerobic conditions.

Received for publication, May 15, 1922.

¹ Studies in Infectious Abortion, 1922.

² Technical Bulletin 49, Mich. Agric. College, Exper. Station, 1921. Cornell Veterinarian, 1921, 11, p. 210.

The method of sealing the inoculated tubes with paraffin, originally described by Holth, has given us fairly uniform results. Sealing wax has also been employed for closing the tubes. Various kinds of medium have been inoculated and the resulting growths compared. It is not considered necessary to give the details of the numerous experiments and therefore only the results are stated.

Following the report of Huddleson the CO_2 method was employed. Certain difficulties were encountered. The gas given off by the generator described by this author contained a considerable amount of chlorin which had an inhibiting effect on the growth of the abortion germs. Also, the generator is not convenient to use, because of the short time during which the reaction goes on and during which the gas is given off. We have used in place of the generator, a cylinder of commercial CO_2 with much greater success. The medium which seems to give us the best results is beef infusion agar. This should be made from lean beef and 2% agar added. It should be adjusted to a P_H of 6.8 to 7.2. We have found a slightly more alkaline medium to be better for the growth of *Bact. abortus* Bang than heretofore reported. At the time this medium is to be used, approximately 10% naturally sterile horse serum should be added to the melted agar, cooled to 50 C., and the tubes allowed to solidify in a slanting position.

These tubes are heavily seeded with the material to be cultivated and then placed in a round Withal Tatum museum jar, the capacity of which is known. The ordinary rubber washer furnished with these jars will not last, as they soon press out. Reinforced rubber diaphragm sheet packing $\frac{3}{16}$ in. thick has been used continually for more than a year with good results. The washer made from this material should be liberally smeared with lanum (Merck) in order to make an air tight connection between the top of the jar and cover.

The inoculated tubes are placed in the jar and with the cover raised only sufficiently to allow the rubber tube (8, fig. 1) from the carbon dioxide tank (1, fig. 1) to enter and approximately 10% of the gas added. The rubber tube is removed quickly, the cover screwed down tightly and placed at 37.5 C. After 24 hours' incubation small pin point colonies will be noted on the medium by observation through the glass walls of the jar. Forty-eight hours' incubation shows well developed colonies of *Bact. abortus*.

Huddleson states that carbon dioxide is directly responsible for the acceleration of the growth of *Bact. abortus*. Up to the time of the publi-

cation of this work it was believed that reduced oxygen tension was the particular factor necessary for the development of this germ. In order to test the specificity of carbon dioxide, duplicate cultures were made and placed in a jar containing 10% hydrogen and others in 10% carbon dioxide. The two groups of cultures were compared at periods of 24, 48 and 72 hours. No difference in the vigor of the

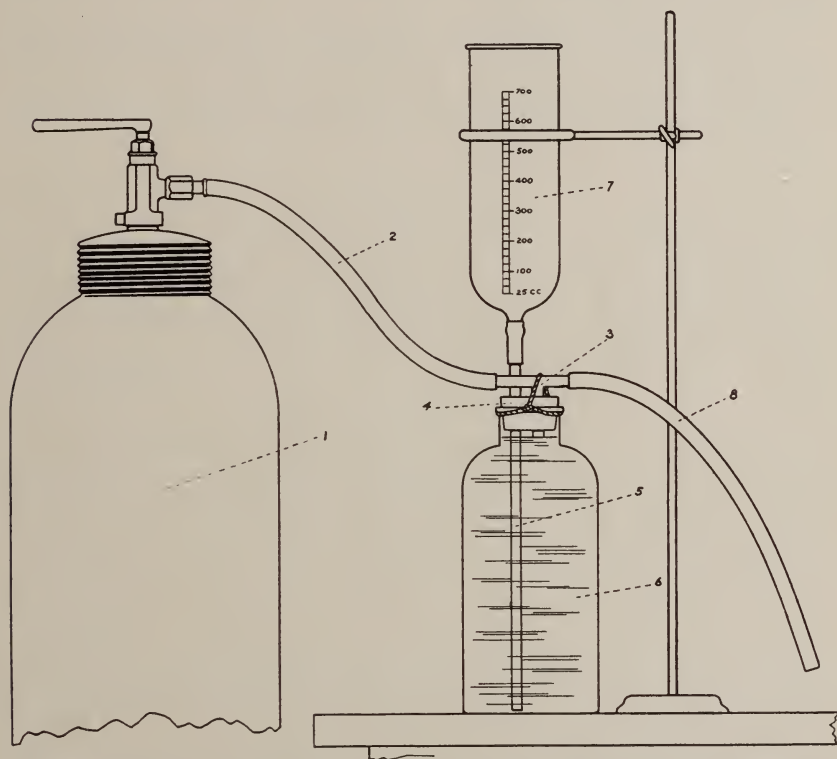


Fig. 1.—Apparatus for the addition of carbon dioxide or hydrogen for the cultivation of *Bact. abortus Bang*; 1, gas tank; 2, rubber tube; 3, glass T tube; 4, two hole rubber stopper (tied in); 5, glass tube leading to bottom of bottle; 6, bottle filled with water; 7, graduated open receptacle for measuring amount of water displaced by the gas; 8, tube leading to the jar in which the cultures are placed.

growth or the microscopic appearance of the organism was noted. It therefore does not seem likely that carbon dioxide exerts any specific action on the growth of *Bact. abortus*. This was also suggested by Edwards³ in his review of Huddleson's article. He states "Beyond showing that an atmosphere in which a certain rather high percentage

³ Vet. Record, 1922, 2, p. 31.

of the gases normally present has been displaced by CO_2 is favorable for the growth of *B. abortus*, Huddleson does not appear to have proved that this gas has any specific properties."

CONCLUSIONS

Horse serum beef infusion agar with a P_H 6.8-7.2 is excellent for cultivating *Bact. abortus* Bang.

The serum agar cultures develop rapidly in an atmosphere of 10% carbon dioxide and hydrogen.

This method of growing *Bact. abortus* has given us our most uniform and satisfactory results.

THE MEIOSTAGMIN REACTION

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Physicochemical reactions undoubtedly play an important part in the field of immunity. However, the finer physicochemical methods have been drawn on but little. The need of an accurate, yet simple laboratory procedure for the diagnosis of malignant disease has long been appreciated. The meiostagmin reaction was introduced to supply this need.

DEFINITION

The meiostagmin reaction (meion, small; stasso, drop) is the name given to a phenomenon which involves a lowering of the surface tension during incubation, when a diluted serum, containing certain antibodies, is mixed with its specific lipoid-containing antigen. The surface tensions of the solutions used were formerly measured by means of the Traube stalagmometer.¹

REVIEW OF THE LITERATURE²

The first work on the meiostagmin reaction was done by Ascoli,³ who determined the surface tension lowering that occurred when an alcoholic extract of typhoid bacilli, properly diluted, was mixed with a diluted typhoid immune serum, in the proportion of 1 part of the former to 9 of the latter. The surface tension was determined immediately after mixing, and again after 2 hours' incubation at 37 C. Normal serum, used in the same dilutions as the immune serum and mixed with the same alcoholic antigen, served as a control.

The alcoholic antigen was prepared as follows: A washed, agar grown culture of *B. typhosus* was first allowed to undergo autolysis by heating in salt solution, and was then passed through a Berkefeld filter. Part of the filtrate was evaporated to dryness, and from the residue a saturated alcoholic extract was made. This was employed as antigen in Ascoli's first work and was found to be more potent than (a) the filtrable aqueous products of autolysis, (b) the precipitate obtained by adding alcohol to (a), (c) the filtrate obtained in the preparation of (b). Various dilutions of the antigens were used, ranging from 1:100 to 1:100,000. With a 1:100 dilution of the antigens, and a 1:20 patient's serum the surface tension lowering during the 2 hours' incubation caused the drop count to increase 3.2 drops, from a stalagmometer delivering 60 drops of water. Greater dilutions of the antigens caused increases of less than

Received for publication, March 20, 1922.

¹ Arch. f. d. ges. Physiol. (Pflüger's Archiv), 1908, 123, p. 419.

² This review merely mentions the articles in chronologic order, stating in each any radical changes in recommended technic. The first two articles, however, are discussed at greater length. A more detailed review of the literature up to 1911 is given by Bernstein and Simons, *Am. Jour. Med. Sc.*, 1911, 142, p. 852.

³ München. med. Wehnschr., 1910, 57, p. 62.

2 drops, while control tubes containing (a) normal saline as antigen and (b) normal serum added to the typhoid extract, gave increases of one drop in each case. This work was done with a typhoid immune serum which agglutinated its specific antigen in a dilution not higher than 1:80 (macroscopically). On a basis of his experiments, Ascoli concluded that an increase of 2 or more drops warranted a diagnosis of typhoid fever.

Shortly after the appearance of Ascoli's first paper dealing with the meiostagmin reaction, one of his co-workers, Izar,⁴ wrote on the same subject. Izar prepared an antigen from the spleen of a syphilitic fetus by simply extracting the desiccated tissue with absolute ethyl alcohol. The alcoholic extract obtained by repeatedly treating 5 gm. of the spleen was finally concentrated to a volume of 10 cc, and of this solution dilutions were made and employed. Specimens of serum from syphilitic patients were tested with this antigen, the dilution of the serums being 1:20, and that of the antigen 1:100. All dilutions were made with normal saline. As controls, these tests were made: (a) All serums were examined in parallel tests, but using alcoholic solutions of tubercle bacilli, carcinoma, echinococcus, and a watery solution of *B. typhosus* as antigens, in dilutions varying from 1:20 to 1:100; (b) non-syphilitic serums were employed using syphilitic antigen. No nonsyphilitic serum gave an increase greater than 1 drop. In no case was there an increase of more than 1.5 drops when known syphilitic serums were tested against heterologous antigens. Two of the "Wassermann positive" serums, however did not give the usual 2 to 3 drop increase, but these were later shown to come from cases of leprosy rather than syphilis. On extending his field of investigation, Izar found that an alcoholic extract of a syphilitic liver was as potent in causing a surface tension lowering as was the alcoholic solution of spleen tissue, but that a similar extract of normal human liver, or of guinea-pig heart was not satisfactory. The work of Izar was thought to indicate, then, that the specific immune body, the so-called meiostagmin, was (a) highly specific, (b) a body of the first order (in Ehrlich's conception of immunity) and (c) that complement was not necessary for its activation, although the presence of complement did not interfere with the reaction.

In a third article by Ascoli and Izar,⁵ the question of adaptability of the reaction to the diagnosis of certain malignant tumors was studied. As antigens they employed the ether soluble portion of evaporated alcoholic extracts of microscopically diagnosed tumors. Only a small percentage of the cases which were examined gave positive reactions, even when the serum of the person from whom the tumor was removed was mixed with an antigen which had been prepared from that tumor. After this preliminary investigation an easily transplantable rat sarcoma was chosen for study. The solvents used in the preparation of the antigen were the same as those mentioned. Blood serum from patients suffering from various diseases, and that of rats to which the sarcoma employed in the preparation of the last mentioned antigen had been transplanted, were all examined, in dilutions varying from 1:20 to 1:50—the dilutions of the antigen were 1:100 and 1:500. The serums of the diseased rats caused an increase in the drop count of from 3 to 5 drops, while all other serums, including normal ones for control, gave increases of less than 1.5 drops. Antigens were prepared from 6 human carcinomas, and these, when tested against the serum of patients known to be suffering from carcinoma, gave increases in the drop count varying between 4 and 8 drops. The authors concluded that perhaps the reaction cannot be employed in the diagnosis of all tumor growths, that some of the tumors may not be suitable for

⁴ *Ibid.*, p. 182.

⁵ *Ibid.*, p. 403.

the preparation of antigens, and that the degree of surface tension change is of minor importance, just so it is greater than the experimental error, and greater than that which occurs in the control tubes.

Later, Izar and Usuelli⁶ elaborated considerably on the details of the various steps in the meiostagmin reaction, and specifically stated certain essential points. I shall repeat at this point some of those which are in direct contradiction of earlier statements made by one of the authors.

(a) First count the diluted serum alone, then add the diluted antigen, incubate at 37 C. for 2 hours. This represents a variation, in that previously the first count was made immediately after addition of the antigen to the serum, rather than before this addition.

(b) The proper strength of the antigen must be arrived at by titration of various dilutions of it. Previously Izar⁴ mentioned the fact that a syphilitic antigen could be used in any dilution, from 1:25 to 1:100,000, but here he insists on the necessity of a preliminary titration.

(c) An increase of two or more drops is a positive reaction. This is too broad a statement, since the number of drops varies inversely as the area of the dropping tip, and therefore the number of drops per unit volume is smaller from stalagmometers having the larger tips. It is mechanically impossible to produce suitable dropping tips with absolutely flat surfaces, and still have them all of the same diameter. In view of this possible variation in the number of drops per unit volume of the same liquid at the same temperature, and the fact that some of Izar's control tubes showed increases of 1.7 drops, the setting of an arbitrary limit of 2 drops' increase, and calling any increase above this a positive test, is unwarranted.

(d) Alcoholic extracts of normal guinea-pig hearts and of normal human liver are here reported as suitable for antigens in the diagnosis of syphilis. In a previous article Izar⁴ specifically stated that these solutions were not suitable as antigens.

The serums of some of their syphilitic patients gave negative meiostagmin reactions, while in some cases positive reactions were obtained with the serum of nonsyphilitic patients. Further work was also reported by Izar and Usuelli on the diagnosis of human and rat sarcomas, in which it was shown that serums reacted not only with homologous antigens, but also with heterologous ones, although to a weaker degree.

Diluted whole blood instead of blood serum was recommended by Izar⁷ in his next publication. This work dealt, for the most part, with diagnosis of certain infectious diseases, such as typhoid fever and tuberculosis. In testing serum from advanced cases of tuberculosis he found that 39 of 40 gave positive reactions. All controls were negative. Of 9 typhoid fever cases giving positive agglutination, all reacted positively with their homologous antigens. This point is mentioned here because attempts to confirm these findings failed utterly, and will be discussed later.

Ascoli and Izar⁸ later immunized animals against normal horse serum, Witte's peptone, and gelatin. After several injections, the serum of these animals was able to react with antigens prepared in the usual way from these substances. These reactions were found to be specific.

After this long series of apparently brilliant results, D'Este⁹ reported his findings in 19 cases of tuberculosis, 4 of them pulmonary tuberculosis, and 15 cases of surgical tuberculosis. The latter were confirmed by operation.

⁶ Ztschr. f. Immunitätsf., 1910, 6, p. 624.

⁷ München. med. Wchnschr., 1910, 57, p. 842.

⁸ Ibid., p. 954.

⁹ Berl. klin. Wchnschr., 1910, 47, p. 879.

In the pulmonary cases, the increases ranged from 1.3 to 3 drops, while in the surgical cases, the range was smaller, extending from 1 to 2.5 drops increase. In testing his antigen against normal serum, the maximum increase in the number of drops was 1.1. In twelve cases of malignant tumors, he obtained increases varying from 1 to 3 drops. It is evident from this summary of his findings that there is an overlapping in positive and control drop increases, and that the findings are not as "decidedly positive" as were those of the earlier workers.

Micheli and Cattoretti,¹⁰ working under Ascoli, on the question of the nature of the specific substance present in the antigens which they were employing, came to these conclusions: (a) The active substances which are extracted from tumor growths, and which are responsible for the specificity of the meio-stagmin reaction are lipoidal in nature, and are thermolabile; (b) not every tumor contains substances of this nature; (c) every antigen must be titrated before use in order to determine the weakest dilution that would cause an increase in the drop count amounting to 1 to 1.5 drops when mixed with diluted (1:20) normal serum. Antigens used in concentrations stronger than this react positively with normal serums also; (d) the active substances in the serums of tumor cases are relatively thermostabile.

In attempting to repeat the work of Ascoli, Verson¹¹ was able to secure positive results in only 10 of 18 cases of malignant tumors. Using the same antigen and serums from 6 nonmalignant cases, the readings were all negative.

Stabilini,¹² also working with malignant and nonmalignant tumors, found that of 32 cases of the former, all gave increases varying from 2.1 to 3 drops, while in 27 cases of nonmalignant growths, and other diseases, the increase never rose above 1.1 drops. He regards an increase of 1.5 to 2 drops as merely suspicious and not as a positive diagnosis.

The first attempt to employ the meiostagmin reaction in differentiating between the various types of tubercle bacilli was probably made by Gasharrini,¹³ who infected rabbits and guinea-pigs with human, bovine and avian strains, and was able to obtain positive meiostagmin reactions with the serums of these animals and antigens prepared from homologous strains within a week after injection of the organisms. Working out the same plan with other organisms, Vigano¹⁴ obtained positive reactions with the serums of 6 typhoid fever cases, using as antigens the alcohol soluble antigen prepared from suspensions of *B. typhosus*. The same serums, when tested against similarly prepared extracts of *B. paratyphosus* A and B, were all negative. I shall discuss this at a later time.

Further changes in technic were advocated in the latter part of 1910, by Ascoli and Izar.¹⁵ The two greatest changes were (1) the preparation of antigens by extracting desiccated pulverized tumors with methyl alcohol instead of using ethyl alcohol and moist tumors, and (2) the use of water instead of normal saline in the dilution of the antigen. No reason for the change in the method of preparation of the antigen was stated. One would suppose that such changes were uncalled for in view of the uniformly good results which these and certain other workers had obtained with antigens prepared in the original way. Likewise, no reason is given for changing from normal saline to water as diluting fluid for the antigens. They specifically state that before incubation only the control tube,

¹⁰ München. med. Wehnschr., 1910, 57, p. 1122.

¹¹ Wiener klin. Wehnschr., 1910, 23, p. 1102.

¹² Berl. klin. Wehnschr., 1910, 47, p. 1498.

¹³ München med. Wehnschr., 1910, 57, p. 1688.

¹⁴ Ibid., p. 1687.

¹⁵ Ibid., p. 2129.

containing 9 c.c. 1:20 serum and 1 c.c. normal saline, is counted, and that after incubation the tube containing the solution which is being tested is counted. Since the diluent of the antigen is not isotonic with the diluent of the serum, the foregoing procedure is not scientific. The surface tension of normal saline is greater than that of distilled water, and the effect, then, of adding the former to the first solution to be tested and the latter to the one which is tested only after incubation, would be to increase the difference between the readings, or, in other words, to make a "positive result more positive."

In the same year Ascoli¹⁶ tested the reaction in the foot and mouth disease of cattle, and obtained positive results in 22 of 28 cases, while only 2 of 35 animals which were not infected reacted positively.

Somewhat later in the same year Micheli and Cattoretti¹⁷ were unable to verify the work of Ascoli on malignant tumors. In working with rabbits which had been immunized against *B. typhosus*, they were able to obtain only negative results. With syphilitic serum, their results were positive against syphilitic antigens, but the serums from tumor cases also reacted positively with the same antigens.

Equally disappointing were the results obtained by Kelling¹⁸ who obtained positive results with the meiostagmin reaction in only 47% of the cases of malignant tumors which came under his observation. Kelling considered any increase in drop count above 1.5 drops as positive.

Bernstein and Simons¹⁹ never obtained what they considered a positive result. They introduced changes in technic as these were reported in the literature. Clinical cases of typhoid fever, presenting positive Widal tests, gave increases in the drop count which were no larger than those obtained by mixing the diluted patient's serums with normal saline instead of diluted antigen. Their counts were made on the solutions under test, and on the control tubes, both before and after incubation (2 hours at 37 C.). Cross tests were also made, using a known typhoid serum, a known syphilitic serum, a typhoid antigen and a syphilitic antigen. All of the tests except one showed an increase of 1 drop. This one showed a decrease of 0.5 drop. Normal salt solution, incubated in the same way, however, also showed a rise in count amounting to 1 drop. With malignant tumors their results were equally disappointing. Decided increases in the drop count were observed only when the antigens were employed in great concentration, but, as they say, "these are to be disregarded, since all investigators are agreed that antigens when used in a dilution of less than 1:50 give unreliable results, and may react strongly positive with normal serums." They conclude that in their hands the meiostagmin reaction has not proved satisfactory as a diagnostic aid.

Burmeister,²⁰ working with carcinomas, was unable to obtain such brilliant results as were certain of the previous workers. He experienced great difficulty in the preparation of suitable antigens.

Since the time of Burmeister's work no further work involving anything new has been reported in the literature on the question of the meiostagmin reaction. This does not mean, however, that no work has been done on this subject. One of the lamentable things in modern science is that masses of negative work remain unpublished, and that by this attitude of silence, later workers are not prevented from following "blind trails."

¹⁶ Deutsch. med. Wchnschr., 1910, 36, p. 1997.

¹⁷ Wiener klin. Wchnschr., 1910, 23, p. 1555.

¹⁸ Ibid., 1911, 24, p. 90.

¹⁹ Am. Jour. Med. Sc., 1911, 142, p. 852.

²⁰ Jour. Infect. Dis., 1913, 12, p. 459.

PROBLEM

In view of the failure of physicians to make use of the method of diagnosis first advocated by Ascoli and Izar, and in view of the absence of later accurate confirmatory work in the field, the feeling of doubt expressed by Zinsser²¹ in these words still exists, "So far experience with the meiostagmin reaction has not been very extensive; not all observers have been able to obtain results as apparently reliable as those of Ascoli and his collaborators. It is not possible, therefore, to express a final opinion regarding this method of investigation." The questions that arise are: (a) Is the method a simple and reliable one? (b) Is the reaction specific, and if so does this specificity depend on the presence of lipoidal materials in the antigens employed? Concerning the second question, it may be stated that Kleinschmidt²² could not produce antibodies with the streptothrix "nastin" of Much; Thiele²³ showed that lipoids possess no specificity and hence cannot give rise to antibodies. Pick and Schwarz,²⁴ however, found that the presence of certain lipoids increased the antigenic power of certain bacteria. This may explain why the ethereal extracts of red blood cells, used by Bang and Forsmann²⁵ caused the elaboration of specific hemolysins—probably by the action of the lipoids in increasing the production of antibodies, the true antigen being the traces of protein present in the extracts. It has been shown by Huntoon, Masucci and Hannum,²⁶ and by Krumwiede and Noble,²⁷ that antibodies are not lipoidal in composition. It is possible, then, that the specificity of the meiostagmin reaction, if it has such, is dependent not on the presence of extractable lipoids in the antigens employed, but rather on the protein material which may be present as "impurities."

PROCEDURE

1. *The Antigens.*—In this work a series of antigens were employed, all but two of which were extracts of agar-grown cultures of bacteria. These were numbered as indicated, and only the numbers are referred to in the tables which follow: (1) the salt solution (0.85% NaCl) soluble products of autolysis of the bacterial cells; (2) the absolute ethyl alcohol soluble portion of (1); (3) the salt solution soluble (0.85% NaCl),

²¹ *Infection and Resistance*, 1919, p. 538.

²² *Berl. klin. Wchnschr.*, 1910, 47, p. 57.

²³ *Ztschr. f. Immunitätsf.*, 1913, 16, p. 160.

²⁴ *Biochem. Ztschr.*, 1909, 15, p. 453.

²⁵ From Jobling, *Jour. Immunol.*, 1916, 1, p. 491.

²⁶ *Ibid.*, 1921, 6, p. 185.

²⁷ *Ibid.*, p. 201.

alcohol insoluble portion of (1); (4) the ether soluble portion of a mortar-ground mass of bacteria (in the preparation of this antigen petroleum ether (ligroin) B.Pt. 30-40 C. was used); (5) the acetone insoluble ether soluble portion of a bacterial mass; (6) the di-ethyl ether soluble portion of a bacterial mass; (8) the chloroform soluble portion of a bacterial mass; (10) the absolute methyl alcohol soluble portion of a bacterial mass; (11) the absolute methyl alcohol soluble portion of (1); (12) the absolute methyl alcohol soluble portion of the liver of a rabbit immunized against *B. paratyphosus* B. This rabbit was killed when the titer for the homologous antigen was 1:800 and that for the heterologous antigen *B. suipestifer* 1:400. (13) The absolute ethyl alcohol soluble portion of the same liver which was used in the preparation of (12).

Antigens 1, 2 and 3 were prepared by scraping off the growth of 20 heavily seeded agar plates of the organisms used, suspending the growth in an excess of normal saline, and washing 3 times by centrifugalization. The washed cells were again suspended in normal saline and were incubated thus for 48 hours at 37 C. to insure autolysis. The mixture was then filtered through a Berkefeld filter, evaporated to dryness, and the residue extracted as follows: for antigen 1 a saturated solution in normal saline was made; for antigen 2 a portion of the residue was extracted 3 times with warm absolute ethyl alcohol, the alcoholic extracts were combined, evaporated to dryness and the residue thus obtained was taken up in the smallest amount of alcohol that would dissolve it; for antigen 3 the alcohol insoluble residue of the evaporated products of autolysis was extracted 3 times with normal saline, the extracts were evaporated to dryness, and the residue thus obtained was taken up in the smallest quantity of normal saline necessary to dissolve it. Antigens 4, 6, 8 and 10 were all prepared from masses of bacteria which had been washed 3 times with normal saline, by centrifugalization. The only difference in the preparation of these 4 antigens was in the solvent used. The washed bacterial mass in each case was ground in a mortar with a small amount of the solvent; the mass was then extracted 3 times with the warm solvent; the combined extracts were evaporated to dryness, and the residue in each case was taken up in the smallest amount of its respective solvent that would dissolve it. Antigen 5 was prepared by dissolving in ether the acetone insoluble portion of a washed bacterial mass and concentrating the extracts as in the case of the other antigens. Antigen 11 was prepared from (1) just as (2) was, absolute methyl alcohol being used

as the solvent instead of absolute ethyl alcohol. Antigens 12 and 13 were prepared by cutting into small pieces the liver of a healthy B paratyphosus B-immune rabbit, washing the tissue with normal saline, and then extracting separate portions of it with absolute methyl and absolute ethyl alcohol, 3 times for 24 hours at 37 C. The methyl alcohol extracts were combined, evaporated to dryness, and taken up in the smallest amount of absolute methyl alcohol that would dissolve the residue. The same procedure was followed in the case of the ethyl alcohol extracts, except that in this case absolute ethyl alcohol was used as the final solvent. These antigens were employed in dilutions ranging from 1:10 to 1:10,000 as indicated in the tables which follow. All dilutions were made with physiologic sodium chlorid solution, which was prepared from chemically pure salt and triply distilled so-called "conductivity water." In all determinations 1 part of the diluted antigen was thoroughly mixed with 9 parts of the diluted blood serum. The same diluent was used for the serum as for the antigen. The surface tension of the mixture was determined before and after incubation.

2. *Obtaining the Blood Serum.*—In this work only the blood serums of rabbits were used. Four of the rabbits, 8, 9, 12 and 13, had previously been immunized against B. paratyphosus B, while 28 served as a normal control. These animals were bled from the heart by means of chemically clean all glass aspirating syringes. In order to avoid the possibilities of irregularities in readings due to possible traces of oil on the skin of the animals, the latter were first clipped closely on the left thoracic wall, and then by means of a pair of small curved dissecting scissors a small "button hole" was cut into the skin. The needle was inserted through this opening. The needles were kept in absolute ethyl alcohol when not in use. The alcohol was burned off immediately previous to the time of use. The blood was transferred from the syringe to chemically clean test tubes, in which it was first allowed to clot, and was then centrifuged. The clear serum was withdrawn with chemically clean pipets, and all dilutions were made in equally clean glass stoppered volumetric flasks of 100 c c capacity. The serums were used in dilutions ranging from 1:10 to 1:10,000.

3. *Surface Tension Apparatus.*—In this series of experiments the actual surface tension was measured in each case, and appears in the tables in dynes. Heretofore all reported work on the meiostagmin reaction was done with the stalagmometer, and the results were tabulated in "drop counts." In some work which I did almost a year ago, using alcoholic extracts of B. typhosus as antigens and diluted typhoid

immune rabbit serum as the liquid to be tested, I employed the accurate though cumbersome and slow working drop-weight apparatus²⁸ in my determinations. All of my results were negative, i. e., the differences between the readings before and after incubation were not greater than the limits of experimental error. Since the time consumed in making a single determination by this means varied from 50 to 90 minutes, there was reason to suppose that perhaps all possible surface tension lowering had taken place before the time of completion of the first determination—before incubation. For that reason, all determinations recorded in this paper were made with the Du Nouy tensiometer²⁹ which is reliable, simple, accurate, and rapid. All of my determinations were not made at the same temperature, but the surface tension was in each case reduced by calculation to what would have been at 22 C., the temperature at which most of the readings were taken. This was done so as to render the results comparable.

4. *Method of Making the Tests.*—As stated, the solutions were employed in the proportion of 1 part of the diluted antigen to 9 of the diluted serum. The surface tension was determined, after thorough mixing, and the transferring of about 2 c c of the mixture to a Syracuse watch glass, by means of a pipet. The remainder (about 3 c c in each case) of the liquid and the pipet were incubated in a water bath at 37 C. for one and a half hours. After incubation the tube and pipet were cooled to 22 C. in a water bath, and the surface tension was again determined. In order to insure the cleanliness of all the apparatus which was used, each piece was first boiled in a strong soap solution, then rinsed thoroughly in running tap water, immersed for upward of half an hour in a sulphuric acid-potassium bichromate cleaning solution, rinsed 3 times in (a) tap water, (b) distilled water, and (c) conductivity water. One of the tables indicates the effectiveness of this treatment.

At the beginning of this work it was noticed that a spontaneous decrease took place in the surface tension of blood serum-antigen mixtures when exposed to the air in the watch glasses. This was found to be due to substances in the serum, and is a property of normal serums as well as of immune serums. In fact, solutions of pure hemoglobin, or any other substance of large molecular dimensions exhibit the same phenomenon. With the serum mixtures, this decrease amounted

²⁸ Jour. Am. Chem. Soc., 1916, 41, p. 499; 38, p. 246.

²⁹ Jour. Gen. Physiol., 1919, 1, p. 521.

to from 1.4 to 2.2 dynes in the first 2 minutes, exposure. Because of these facts I always waited for from one to one and one-half minutes before taking readings, and then averaged the fourth, fifth and sixth, disregarding the first 3.

The following tables and discussion summarize the results of over 1,100 determinations which were made on almost 200 different antigen-serum mixtures, and which are characterized by one thing in particular, namely, the uniformity of negative findings with my 4 immune serums and all of my antigens.

The serums used in the tests were from rabbits that had been immunized against the specific organism from which the antigens were prepared. The titer of the serums were above 1:6,000 (macroscopic

TABLE 1
RESULTS OF TESTS WITH SERUM OF RABBITS IMMUNIZED AGAINST THE SPECIFIC ORGANISM
FROM WHICH ANTIGENS WERE PREPARED *

Antigens	Rabbit Serum 8			Rabbit Serum 12		
	Before	After	Difference	Before	After	Difference
B. paratyphosus B (1) 1:100.....	77.9	78.4	+0.5	78.2	77.9	-0.3
B. paratyphosus B (1) 1:1,000.....	78.6	78.2	-0.4	78.2	78.1	-0.1
B. paratyphosus B (1) 1:10,000.....	78.3	77.8	-0.5	78.2	77.9	-0.3
B. paratyphosus B (8) 1:100.....	69.3	68.3	-1.0	78.2	78.3	+0.1
B. paratyphosus B (8) 1:1,000.....	69.2	67.7	-1.5	78.2	78.3	+0.1
B. paratyphosus B (8) 1:10,000.....	69.6	67.7	-1.9	78.2	78.2	0.0
B. paratyphosus B (6) 1:100.....	78.3	77.9	-0.4
B. paratyphosus B (6) 1:1,000.....	78.3	77.8	-0.5
B. paratyphosus B (6) 1:10,000.....	78.1	77.9	-0.2

* The numbers in parentheses after the name of the antigen indicate what the extractive was in each case ("Preparation of Antigens"). Next comes the dilution of the antigen, then a figure representing the average of three surface tension determinations made before the time of incubation, another figure representing the same thing for determinations made after incubation, and finally, the difference (in dynes) between these averages.

agglutination). In the reports of the Italian workers increases of over 2 drops in the drop counts were considered positive. The liquids employed were such that the drop counts were between 56 and 60 drops from a stalagmometer delivering 60 drops of water at 20 C. Since the total volume of liquid delivered was constant, and since the density was constant, the surface tension must have varied inversely as the number of drops, hence since the surface tension of water at 20 C. is about 78 dynes (28), and that of the antigen serum complex was nearly that, an increase of over 2 drops would indicate a surface tension lowering of over 1.6 dynes. On that basis all but one of the readings in table 1 are negative, and even in the case of that one, a parallel determination on the serum of rabbit number 12 was distinctly negative. The serum in the foregoing series was used in a 1:10,000 dilution.

According to a previously cited report, the antigens must be titrated before use as in the higher concentrations they will act positively even with normal serums. Table 2 contains a series of determinations which were made in an attempt to confirm this statement.

TABLE 2
RESULTS OF TESTS MADE WITH SERUMS TITRATED BEFORE USE

Serum Dilutions	Surface Tension		
	Before	After	Difference
1:10.....	61.8	60.5	-1.3
1:20.....	63.3	63.3	0.0
1:40.....	65.4	65.2	-0.2
1:80.....	65.4	65.2	-0.2
1:100.....	65.3	65.2	-0.1
1:1,000.....	75.9	75.7	-0.2
1:10,000.....	77.9	78.4	+0.5

* Antigen, *B. paratyphosus* B 1, dilution 1:10; serum from *B. paratyphosus* immune rabbit 8.

TABLE 3
RESULTS WITH SERUMS OF TWO RABBITS IMMUNIZED AGAINST *B. PARATYPHOSUS* B, AND ONE NORMAL RABBIT *

Antigens	Rabbit 8			Rabbit 12			Rabbit 28 (Control)		
	Before	After	Difference	Before	After	Difference	Before	After	Difference
<i>B. parat. B</i> (1) 1:20	63.3	63.3	0.0
<i>B. parat. B</i> (1) 1:20	63.9	63.6	-0.3
<i>B. parat. B</i> (1) 1:100	67.9	67.8	-0.1	67.0	67.0	0.0	66.4	66.8	-0.4
<i>B. parat. B</i> (10) 1:100	66.8	67.3	+0.5	66.4	67.3	+0.9	64.9	65.9	+1.0
<i>B. parat. B</i> (10) 1:1,000	68.3	68.7	+0.4	66.6	66.8	+0.2	65.2	67.0	+1.8
<i>B. parat. B</i> (11) 1:100	66.5	67.3	+0.8	65.6	66.1	+0.5	66.2	65.6	-0.6
<i>B. parat. B</i> (11) 1:1,000	67.0	66.3	-0.7	66.4	66.7	+0.3	65.6	65.8	+0.2
<i>B. typh. Hop.</i> (2) 1:100	66.2	68.2	+2.0	64.7	64.6	-0.1
<i>B. typh. Bari.</i> (2) 1:1,000	67.8	68.0	+0.2	64.7	64.6	-0.1	66.4	66.7	+0.3
<i>B. dys., Flex.</i> (2) 1:100	67.4	67.7	+0.3	64.2	64.2	0.0	67.7	66.1	-1.6
<i>B. dys., Shig.</i> (2) 1:100	67.7	66.8	-0.9	64.1	63.6	-0.5
<i>B. suipestifer</i> (2) 1:100	68.3	68.3	0.0	63.1	63.1	0.0	66.7	66.7	0.0
<i>B. suipestifer</i> (4) 1:100	67.4	67.2	-0.2	64.9	63.4	-1.5	67.4	66.4	-1.0
<i>B. suipestifer</i> (6) 1:100	67.3	66.3	-1.0	63.0	62.8	-0.2
Control. salt solution plus dil. serum.....	69.9	68.9	-1.0

* In table 3 and in those which follow: *B. parat. B* stands for *B. paratyphosus* B strain 12, *B. typh. Hop.* for *B. typhosus* (Hopkins), *B. typh. Bari.* for *B. typhosus* (Bari-more), *B. dys., Flex.* for *B. dysenteriae* (Flexner), *B. dys. Shig.* for *B. dysenteriae* (Shiga) and *B. suipestifer* 118 is represented by *B. suipest.*

There is nothing in this series of determinations to verify the statements referred to.

The results recorded below in table 3 were obtained with 1:20 diluted serums of 2 rabbits which had been immunized against *B. paratyphosus* B and with 1 normal rabbit, 28, which served as a control. In this series antigens prepared from the organism used in the immuniza-

tion of the rabbits, and, in addition, several other heterologous antigens, were employed. Included in the heterologous series were some prepared from *B. suipestifer*, which was agglutinated by the paratyphoid immune serums in almost as high dilutions as was the homologous organism. The dilutions and numbers of the various antigens are recorded as they were in table 1.

Determinations with the same antigens and the serums (1:20) of *B. paratyphosus*-immune rabbits 9 and 13 are not recorded. The results were similar to those tabulated in the foregoing. Serums from the same 5 animals were employed in dilutions of 1:100 and 1:1,000 mixed with homologous antigens 1, 2, 4, 6 and 8 and with the heterologous ones which are recorded in table 3. The antigens were

TABLE 4
RESULTS OF TESTS WITH ANTIGENS 12 AND 13. DILUTION OF SERUMS 1:20 IN ALL CASES

Antigens	Rabbit 8			Rabbit 9			Rabbit 12		
	Before	After	Difference	Before	After	Difference	Before	After	Difference
B. parat. B (12) 1:100	63.4	64.9	+1.5	64.6	64.2	-0.4	64.4	63.3	-1.1
B. parat. B (12) 1:1,000	64.6	66.0	+1.4	61.9	62.9	+1.0	64.6	64.9	+0.3
B. parat. B (13) 1:100	64.4	65.0	+0.6	63.9	62.0	-1.9	66.5	66.9	+0.4
B. parat. B (13) 1:1,000	66.7	66.3	-0.4	64.3	65.5	+1.2	65.8	65.5	-0.3
	Rabbit 13			Rabbit 28 (Control)					
	Before	After	Difference	Before	After	Difference			
B. parat. B (12) 1:100	62.8	63.9	+1.1	63.5	63.9	+0.4			
B. parat. B (12) 1:1,000	65.9	64.3	-1.6			
B. parat. B (13) 1:100	63.5	64.9	+1.5	65.6	65.9	+0.3			
B. parat. B (13) 1:1,000	65.8	64.9	-0.9	65.4	65.1	-0.3			

used in dilutions of 1:100 and 1:1,000 when mixed with the homologous serums and 1:100 only in the cases of the typhoid, dysentery and *suipestifer* antigens. The results of these experiments are not recorded because the differences and small irregularities which were observed were similar in every respect to those of table 3.

This work fails in every way to confirm the findings of the earliest workers with the meiotagmin reaction. Only occasionally were results obtained which were even "suspicious," but other tests in parallel series failed to check up with these cases or normal serums, to which the same antigens had been added, or to which (in two cases) only normal saline had been added, caused equally large changes. The limits of experimental error are as great in the higher dilutions of antigen and serum as in the more concentrated mixtures.

After this long series of disappointingly negative experiments, it was decided to make one final effort to obtain a positive result. A rabbit which had been immunized against *B. paratyphosus* B, and whose serum agglutinated the homologous antigen in a 1:800 dilution, was given an overwhelmingly large injection of a live culture of this organism. The animal died within 6 hours. The liver was removed, and from it antigens 12 and 13, discussed in the foregoing, were prepared. These

TABLE 5

DETERMINATIONS ON "CONDUCTIVITY WATER," SODIUM CHLORIDE SOLUTION AND SALT SOLUTION PLUS ANTIGEN

Determinations on "Conductivity Water"									
Before					After				Difference
Sample 1	105.1	105.2	105.1 = 105.1 = 77.9		105.0	104.9	105.1 = 105.0 = 77.8		-0.1
Sample 2	105.1	105.3	105.4 = 105.3 = 78.1		105.2	105.1	105.1 = 105.1 = 77.9		-0.2
Sample 3	105.1	105.1	105.1 = 105.1 = 77.9		105.1	104.9	105.0 = 105.0 = 77.8		-0.1
Determinations on 0.85% Sodium Chloride Solution									
Before					After				Difference
Sample 1	105.7	105.7	105.7 = 105.7 = 78.3		105.6	105.4	105.7 = 105.6 = 78.3		0.0
Sample 2	105.4	105.3	105.4 = 105.4 = 78.2		105.4	105.4	105.4 = 105.4 = 78.2		0.0
Sample 3	105.6	105.6	105.4 = 105.5 = 78.3		105.5	105.7	105.5 = 105.6 = 78.3		0.0
Determinations on Salt Solution Plus Antigen (In each of the determinations which appear in this section, 9 parts of normal saline were mixed with one part of diluted antigen)									
Before					After				Difference
Sample 1, salt plus <i>B. parat. B</i> (2) 1:100	105.3	105.2	105.3 = 105.3 = 78.1		105.3	105.3	105.3 = 105.3 = 78.1		0.0
Sample 2, salt plus <i>B. parat. B</i> (4) 1:100	105.5	105.6	105.4 = 104.5 = 78.2		105.4	105.3	105.3 = 105.3 = 78.1		-0.1
Sample 3, salt plus antigen <i>B. parat. B</i> (6) 1:100	105.3	105.4	105.3 = 105.3 = 78.1		105.3	105.4	105.3 = 105.3 = 78.1		0.0
Sample 4, salt plus antigen <i>B. parat. B</i> (8) 1:100	105.8	105.7	105.8 = 105.8 = 78.4		105.8	105.7	105.8 = 105.8 = 78.4		0.0
By adding 1 c.c. of normal saline to 9 c.c. of a 1:20 dilution of rabbit serum the following differences were noted									
Before Incubation					After Incubation				Difference
Rabbit 9	88.4	87.9	87.9 = 88.1 = 65.4		86.0	85.6	85.4 = 85.7 = 63.7		-1.7
Rabbit 13	88.9	88.0	88.0 = 88.3 = 65.5		88.4	88.0	87.8 = 87.7 = 65.2		-0.3
Rabbit 28	89.4	89.0	88.9 = 89.1 = 66.1		89.8	89.4	89.1 = 89.4 = 66.4		+0.3

antigens were tested against the potent *B. paratyphosus* B. immune serums of rabbits 8, 9, 12 and 13, and against the normal serum of control rabbit 28. The results of this work are recorded in table 4.

The results here tabulated are more erratic in appearance than those in the earlier tables. The peculiar thing is that deviations occur in both directions, i. e., some indicate increases in the surface tension, while others show decreases. The results are therefore as strikingly negative as those obtained with the other antigens, and correspond to

the findings of Bernstein and Simons,¹⁹ who used positive typhoid and syphilitic serums and typhoid and syphilitic antigens in a series of cross tests. In their work, normal salt solution gave as great a decrease in the surface tension as their antigen-serum mixtures did, and their syphilitic antigen syphilitic serum mixture gave no decrease, while a syphilitic serum typhoid antigen mixture gave an increase.

Because of the irregularity with which surface tension lowering was observed, and also because at times a lowering was observed in one tube while a similar tube in a parallel series showed an actual increase, a set of control tests was run in order to determine these points: (a) the accuracy of the instrument; (b) the presence or absence of impurities on the glassware used; (c) the surface tension changes taking place in pure water and in normal saline during a period of incubation similar to that used in the actual tests; (d) which substances were responsible for the irregularities observed in the antigen-serum mixtures?

The table shows readings as taken in triplicate, the average of the 3, and the surface tension corresponding to this average, both before and after incubation, and the difference if there was any.

From these sets of controls it is evident that the apparatus gives accurate readings, the cleaning of the glassware is adequate, pure water, salt solution, and diluted antigen alone do not give irregular spontaneous surface tension changes during incubation, and that it is the serum which is responsible for the large experimental error.

SUMMARY AND CONCLUSIONS

Over 1,100 tests were made in a study of the meiostagmin reaction, using almost 200 different mixtures of rabbit serum and antigen. The serum was obtained from animals immunized against *B. paratyphosus* B, and the antigens were prepared from the homologous and several heterologous organisms, and from the liver of a healthy *B. paratyphosus* B immune rabbit.

The spontaneous surface tension changes and the limits of experimental error are as great when relatively dilute serum is employed in the test as when the serum is diluted only 1:20. Serum more dilute than 1:1,000 shows a smaller degree of, and a slower rate of, spontaneous surface tension change than lower dilutions. These facts apply to normal as well as immune serums.

The substance in the antigen-serum mixtures which is responsible for relatively large experimental error is the blood serum, as measurements on the various other substances used in these mixtures gave constant and accurate readings.

The Du Nouy surface tension apparatus, not previously commented on by any one but its inventor, gives readings with the biochemical mixtures employed, which are as accurate as those obtainable with the more cumbersome and slow drop weight apparatus. The sources of experimental error are less than those involved in the use of the Traube stalagmometer.

The meiostagmin reaction does not reveal the presence of antibodies in *B. paratyphus* B immune rabbit serum of high titer regardless of (a) the dilutions in which the serums and antigens are employed, and (b) of the solvents used in the preparation of the antigens.

THE EFFECT OF UREA ON IMMUNOLOGIC REACTIONS

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In a previous article¹ I reported that urea inhibited the action of rabbit complement. The present paper contains further results which show the effect of urea on human, guinea-pig, dog and hog complement, on normal dog antihuman hemolysins, and the effect of intravenous injections of urea and sodium chloride, respectively, on rabbit complement and the leukocyte count. These observations are paralleled with blood urea determinations.

The hemolytic system (antihuman) and the technic of studying the effect on complement *in vitro* was similar to that described.¹ The method used in determining the effect on complement *in vivo* of M/1 urea and M/1 NaCl solutions was essentially as follows:

The normal urea content per 100 c c of blood was determined several times in each rabbit. Immediately after the blood was drawn for the last determination 2 c c of M/1 urea were injected intravenously into one rabbit and 2 c c of M/1 NaCl into another. Repeated injections, varying in amount from 2 to 6 c c, complement titrations and blood counts were made approximately every hour. The quantitative determination of urea was essentially that given by Gradwohl² except that the ammonia was drawn over into a known amount of N/100 HCl and the amount of uncombined acid determined by direct titration, using methyl orange as an indicator. From this the amount of urea per 100 c c of blood was calculated.

It was found that complement of different animals differs markedly in its activity in the presence of urea. Human complement was inactive in the presence of 0.02 to 0.04 c c of M/1 urea, rabbit, hog and dog complements in the presence of 0.3, 0.1 and 0.2 c c, respectively, while guinea-pig complement did not seem to be materially affected. Normal dog lysins for human corpuscles as a rule failed to produce hemolysis in the presence of 0.3 to 0.4 c c M/1 urea. In my previous article,¹ I reported that urea did not prevent the union of amboceptor and corpuscles, and I have confirmed this result.

From a number of experiments it seems that the so-called "mid-piece" of complement did not combine with the amboceptor-cell complex within the usual incubation period during which time the controls were completely laked.

Received for publication, May 8, 1922.

¹ Jour. Infect. Dis., 1917, 20, p. 185.

² Newer Methods of Blood and Urine Chemistry, 1917, p. 42.

As regards human complement, this effect is permanent, but in the case of rabbit, hog and dog complement, there is either a permanent interference or a marked slowing of the reaction. In the latter instance, if the tubes are placed in the icebox for 24 hours after the regular 30 minute incubation in the 37 C. water bath, hemolysis frequently results. This fixing or restraining effect did not occur with guinea-pig complement. There is no correlation between the amount of serum constituting a unit of complement and these results. The degree of the restraining effect seems to vary to some extent with the sample of urea. In all, 4 samples of chemically pure urea were used with quantitatively and qualitatively identical results for 3 samples and a slight quantitative difference for the fourth, which gives the slowing rather than the fixing effect observed for other than human complement which apparently underwent permanent "fixation."

It may be that the restraining effect slows down the reaction sufficiently to permit deterioration of complement. One has to consider the possibility of urea not being chemically pure. Mathews³ states that small quantities of cyanamide may be present in urea solutions. In order to determine whether the urea I used contained cyanamide I made use of the qualitative test for cyanamide suggested by Caro, Schuck and Jacoby.⁴ This is based on the observation that the silver salt is an amorphous yellow substance almost insoluble in dilute ammonia. I found this sufficiently sensitive to detect 1 part of cyanamide in 100,000 parts of dilute ammonia water. None of my samples of urea showed any evidence of containing cyanamide. Theoretically, the urea used in these experiments was chemically pure.

The blood urea determinations suggest that there is no correlation between normal urea content and the inhibitory effect of urea on complement. Human complement, which was most easily affected, contains very much less urea than that present in the other complements studied.

This work suggests fundamental differences in the complement of these animals, although this may be more apparent than real. It also indicates a new reason for the superiority of guinea-pig complement over other complements.

The results obtained by the intravenous injections of M/1 NaCl and M/1 urea are suggestive. The complement content compared with blood urea shows that following the injection of NaCl there was no accumulation of urea but instead an initial drop owing, probably, to its diuretic effect. The complement content either remained normal or was

³ *Physiol. Chemistry*, 1916, p. 17.

⁴ Cited by Franke, *Cyanamid*, 1913, p. 20.

increased. On the other hand, when M/1 urea was injected there was an initial drop in complement content associated with an increase in blood urea, followed by a rise associated with relatively low blood urea occurring apparently at the time of maximum activity on the part of the kidneys. Two hours later when the blood urea had risen from 47.3 to 60.2 mg. per 100 c c of blood there was a corresponding drop in complement content. During the next 4 hours the complement did not return to normal. The low complement content seemed to be associated with decreased activity on the part of the kidneys. This result suggests that a study of relation of renal function to complement content might be of value.

In reporting on the effect of any substance on the blood counts of rabbits, I am aware of a great normal variability which tends to lessen the significance of any comparative data. For this reason I would rather merely state results and not draw general conclusions. One injection of 2 c c of either M/1 urea or M/1 NaCl produced practically identical results, i. e., an initial leukopenia followed by a leukocytosis and then a return to normal within an hour or two. Occasionally there was a slight initial rise followed by a drop with a later rise to normal. Repeated injections of M/1 urea produced a leukopenia which persisted, as a rule, during the period of injection and from 1 to 4 hours after the last injection. This does not seem to hold, as a rule, for similar amounts of NaCl.

While Fosse⁵ and others have carried out rather extensive work showing that urea is widely distributed in nature, very little seems to have been done to determine its exact physiologic and pharmacologic action. While it is a normal product, it is not present in normal persons as a rule in amounts much above 25 to 35 mg. per 100 c c of blood. In man, perhaps the most important precursor of urea is ammonia, while in some animals it is formed by oxidation and hydrolysis of uric acid. In man, the mechanism might be considered a protective one against the accumulation of an undue amount of ammonia. Perhaps urea normally has a definite physiologic function for man as cited by Mathews³ for elasmobranch fishes. Von Furth⁶ summarized the work of Heilner on the physiologic action of urea as follows: "Urea introduced subcutaneously has a stimulative effect on protein metabolism, thus suggesting the possibility of urea being a factor in the special mechanism regulative of the course of intracorporeal protein disintegration." Eyster⁷ states that urea has a stimulative action on the heart.

⁵ Ann. de l'Inst. Past., 1916, 30, p. 525.

⁶ Chemistry of Metabolism, 1916, p. 499.

⁷ Science, 1910, 31, p. 236.

These statements would indicate that relatively small doses of urea are apparently either of no effect or produce a stimulative one. As to large doses, Hewlett, Gilbert and Wickett⁸ have shown that 100 gm. of urea given orally to man leads to a rapid rise of blood urea content associated with many of the symptoms of uremia.

The experiments I have reported are added evidence of the toxic effect of urea in the body when given in relatively large amounts and add to the significance of the results obtained *in vitro*. Clinically, in uremia, there has long been noted a lowered resistance to infection associated commonly with leukopenia.

SUMMARY AND CONCLUSIONS

Urea solutions restrain the union of complement with the amboceptor-cell complex.

The slowing effect varies for different complements ranging from permanent fixation for human complement to no appreciable effect for guinea-pig complement.

In test tube experiments it requires 10 times as much M/1 urea to inhibit rabbit and 7 times as much for hog complement and 10 times as much for dog complement as it takes for human complement.

Urea in NaCl solution in the concentrations stated does not directly lase red blood cells or interfere with the union of amboceptor and red blood cells.

The intravenous injection of 2 to 6 c c M/1 NaCl solution in rabbits gave a slight diuretic effect but no noticeable effect on complement.

Repeated injections of similar amounts of M/1 NaCl produced a slight initial leukopenia followed by leukocytosis in rabbits. One injection gave an initial leukopenia lasting for two hours, followed by a return to normal.

Repeated injections of M/1 urea solution was associated with a decrease in complement content.

The slight rise in complement two hours after the initial injection was associated with apparent maximum activity on the part of the kidneys.

Repeated injection of M/1 urea was associated with wide fluctuations in the leukocyte count, but on the whole produced noticeable leukopenia. One injection of 2 c c of M/1 urea gave an initial leukopenia followed by a marked leukocytosis, the blood count returning to normal within three hours and remaining normal.

⁸ Arch. Int. Med., 1916, 18, p. 636.

STUDIES OF HEMOLYTIC STAPHYLOCOCCI

HEMOLYTIC ACTIVITY—BIOCHEMICAL REACTIONS—SEROLOGIC REACTIONS

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I. STUDY OF HEMOLYTIC ACTIVITY

That staphylococci lake blood was brought out in 1900, when Kraus¹ noticed the hemolytic effect of staphylococci on bloodplates. The following year Neisser and Wechsberg² demonstrated a hemolytic substance in filtrates of broth cultures. They found that in alkaline beef broth, this hemolytic substance began to appear on the fourth day and reached a maximum between the eighth and fourteenth day. In a general way they showed that aureus and virulent strains produced greater quantities of hemolysins than did either the albus or avirulent strains. Van derme³ found that the hemolytic power was generally greatest in cultures freshly isolated from pathologic conditions, and was generally absent in cultures from dust and from the normal mouth. Todd,⁴ working with *B. megatherium* and Kraus⁵ working with staphylococcus showed that this action takes place in vivo as well as in vitro.

PRODUCTION OF HEMOLYSIN

It had been observed that in a general way staphylococci would show hemolysis to a greater or less extent on blood-agar plates within 24 hours. In addition, the hemolysis was not typical of an exogenous hemolysin, as is typical of *Streptococcus hemolyticus*; but rather resembled an exogenous product of metabolism, as in the case of *B. coli*, where the hemolysis diffuses haphazardly through the medium.

The first experiment was made to determine what analogy there was in chronicity in the production of hemolysins on blood plates and in broth. It might be stated here that all the work on hemolytic activity was obtained with 4 cultures representative of all the strains studied. Two were known hemolytic, and 2 were originally isolated as nonhemolytic. Twenty-four hour cultures were seeded into 10% horse (inactivated) serum broth in Erlenmeyer flasks and incubated at 37 C. for 24 hours. At the end of each 24-hour period, 5 c c of the culture were removed

Received for publication, May 22, 1922.

¹ Wien. Klin. Wchnschr., 1900, 13, p. 49.

² Ztschr. f. Hyg. u. Infektionskr., 1901, 36, p. 299.

³ Hyg. Rundschau, 1903, 13, p. 66.

⁴ Trans. London Path. Soc., 1902, 53, p. 196.

⁵ Wien. klin. Wchnschr., 1902, 15, p. 382.

aseptically and centrifuged at high speed for 5 minutes. One c c of the clear supernatant fluid was added to 1 c c of a washed 2.5% horse-blood suspension and incubated at 37 C. for 2 hours, at the end of which time the tubes were read for hemolysis. The concentration of blood attempted to approximate as closely as possible the conditions of the blood plate.

It was found that no estimable hemolysins were produced in broth cultures within 24 hours. In fact, as will be borne out later, no hemolysins were shown to be present until the sixth day. It may be that the discrepancy in time between plate and broth cultures is explainable on the grounds that in the former case the hemolysins are so concentrated around each colony as to assert themselves at a conspicuously earlier period; whereas in the latter case the hemolysins go into solution and become too dilute to have any effect on a suspension of blood cells.

The next experiment was planned to obtain the curve for the production of hemolysins. The technic employed was the same as in the preceding experiment, except for one detail. The cultures were seeded into tubes containing 10 c c of the serum broth, and at the end of each day one tube was removed from the incubator and used for the tests. Care was taken to keep the volume of the tubes constant by adding sterile salt solution to repair any loss by evaporation.

Table 1 shows that hemolysins begin to appear on the sixth day, reach a maximum at the ninth and tenth days, and disappear between the thirteenth and sixteenth days.

With the period of hemolysin production established, the logical sequence was to determine if possible the source or the cause of the production. It was assumed entirely theoretically that hemolysis is caused by one of the following or perhaps combination of factors:

1. Reaction: An increase or decrease in hydrogen-ion concentration sufficient to cause hemolysis.
2. Tonicity: An increase or decrease in the tonicity of the medium sufficient to cause crenation or laking of the blood corpuscles.
3. Hemotoxin: A hemolytic substance elaborated and secreted by the bacterial cell, causing hemolysis.
4. Proteolysis: The production by the bacterial cell of some substance for the utilization of the blood protein. Under this head would be included autolytic products also.

In order to establish experimentally which hypothesis was correct the following procedure was adopted: Coincidental with testing for the

presence of hemolysins, the hydrogen-ion concentration was read on the Clark and Lubs ⁶ scale; the amino acidity was titrated by the Sørensen ⁷ method; the proteose content was determined by the Vernon tests; ⁸ and numerical counts made at the end of each day, as long as was deemed necessary for the points at hand.

TABLE 1
PRODUCTION OF HEMOLYSINS

	Days															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Strain A1 (From Air)																
Hemolysis.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amino acidity.....	54	56	56	56	56	54	58	58	50	50	50	50	46	38	36	36
Proteose content.....	0.25	0.25	0.25	0.25	0.25	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
H-ion concentration..	7.9	7.9	8.0	8.1	8.2	8.3	8.3	8.3	8.3	8.3	8.3	8.3	8.4	8.4	8.4	8.4
Strain A5 (From Air)																
Hemolysis.....	0	0	0	0	0	0	±	+	+++	++	+1	+	+	+	±	0
Amino acidity.....	56	64	64	56	84	80	98	102	88	84	84	84	72	72	44	44
Proteose content.....	0.25	0.25	0.25	0.25	0.3	0.3	0.35	0.4	0.4	0.4	0.4	0.45	0.45	0.45	0.45	0.45
H-ion concentration..	7.7	7.9	8.0	8.0	8.2	8.3	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4
Strain H2 (From Heart Blood)																
Hemolysis.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amino acidity.....	54	56	56	56	56	56	54	52	46	44	44	44	38	36	34	34
Proteose content.....	0.25	0.25	0.25	0.25	0.25	0.3	0.3	0.3	0.25	0.3	0.3	0.3	0.3	0.3	0.3	0.3
H-ion concentration..	7.7	7.9	8.0	8.2	8.2	8.2	8.2	8.2	8.2	8.3	8.3	8.3	8.4	8.4	8.4	8.4
Strain T9 (From Throat)																
Hemolysis.....	0	0	0	0	0	0	±	+	++	+	+	+	±	0	0	0
Amino acidity.....	54	56	56	56	56	54	86	74	66	56	56	56	58	44	38	38
Proteose content.....	0.25	0.25	0.25	0.25	0.25	0.3	0.3	0.3	0.3	0.3	0.3	0.35	0.35	0.35	0.35	0.35
H-ion concentration..	7.7	7.9	8.0	8.1	8.2	8.2	8.2	8.2	8.3	8.3	8.3	8.3	8.4	8.4	8.4	8.4
Control.....	Hemolysis 0					Amino Acidity 38				Proteose 0.25				H-ion 7.5		

Figures for proteose content represents amount of medium required to equal 1 cc of standard.

Figures for amino acidity represent cc of 20N NaOH required to neutralize 100 cc of medium.

Plus signs indicate: +, 25% hemolysis; ++, 50%; +++, 75%.

Hours	Bacterial Counts Made With Production of Hemolysin Stains			
	A 1	A 5	H 2	T 9
0.....	40,000	72,000	50,000	73,000
24.....	100,000,000	180,000,000	450,000,000	300,000,000
48.....	830,000,000	200,000,000	850,000,000	1,000,000,000
72.....	1,210,000,000	6,300,000,000	8,900,000,000	9,200,000,000
96.....	7,000,000,000	1,000,000,000	2,500,000,000	2,000,000,000
120.....	460,000,000	300,000,000	3,200,000,000	800,000,000
144.....	150,000,000	350,000,000	800,000,000	600,000,000

1. An analysis of the results (table 1 and chart 1) shows several points: The ultimate reaction of all the cultures—hemolytic and non-

⁶ Jour. Bacteriol., 1917, 2, p. 109.

⁷ Biochem. Ztschr., 1908, 7, p. 45.

⁸ Jour. Physiol., 1904, 30, p. 330.

hemolytic alike—is the same, P_H 8.4. If the hemolysis were the effect of reaction, all the cultures should show a like behavior on blood. But since the cultures do not show the same hemolytic activity, it is reasonable to exclude reaction as the cause of hemolysis. Incidentally, sterile salt solution, the reaction of which is adjusted to P_H 8.4, does not cause hemolysis.

Charts 1 and 2.—Showing counts, amounts of amino acids and hemolytic substances produced.

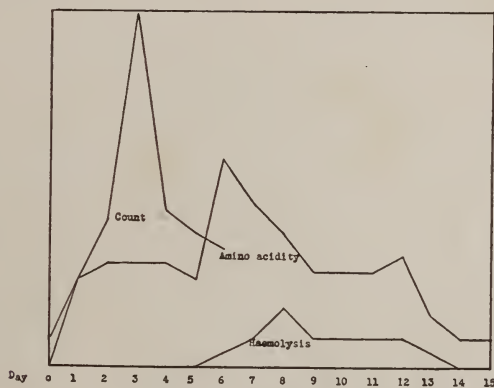


Fig. 1.—Strain T9.

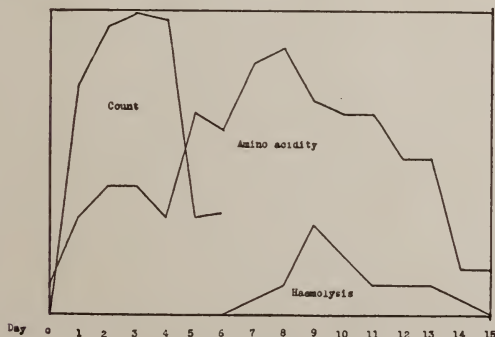


Fig. 2.—Strain A5.

2. No effort was made to determine the tonicity of the cultures. The impression was gathered from the work of Larson et al.⁹ that bacteria of themselves do not change the surface tension of mediums, and in their study specific depressants were added when a drop in surface tension was desired.

⁹ Larson, Cantwell, and Hartzell: Jour. Infect. Dis., 1919, 25, p. 41.

3. The figures for the numerical counts show that there is an increase in the number of staphylococci until the third day, when a maximum is reached. From then on there is a sharp decrease in numbers, indicating that growth of an active nature at least has come to a cessation. If the production of hemolysins and the numerical counts had shown a parallelism, it could have been reasonably assumed that the hemolysin were a true secretion product and a definite hemotoxin. Since they show no such parallelism, however, the hemolysin must be of some other nature.

4. The course of proteolysis or amine acidity runs a definitely parallel course to the curve of hemolysin production. The suggestion offered itself that if not directly associated, then some close relationship must exist between the two. Further study reveals the following concatenation of events: (1) the period of maximum growth occurs on the third and fourth day; (2) the maximum production of amino acidity occurs on the seventh and eighth days; (3) the maximum production of hemolysins occurs on the ninth and tenth days. Stated in another way, the growth period precedes the amino acidity period, which in turn precedes the hemolysin production period. It would seem from such an interrelated process that the production of hemolysins is a proteolytic process and perhaps even autolytic.

There is one other point of interest brought out by this experiment. Although there is an increase in amino acidity, there is no corresponding decrease in proteose content. This is probably due to the fact that the biuret test, used in determining the amount of proteose present, shows the presence of substances other than proteose; so that even if proteose were proteolyzed to form polypeptides, peptides and the higher amino acids the intensity of the color would still remain the same. One other point—it shows that the production of erepsin by staphylococci enables them to attack peptones and proteoses.

5. Following the suggestion offered in the foregoing experiment, the next step was to determine what rôle autolysis plays in hemolysis. For this purpose 24-hour cultures were inoculated in Erlenmeyer flasks (10% serum broth), and incubated at 37 C. for 5 days. This culture was then distributed in equal volumes into test tubes. To one series was added 0.25% phenol, to a second 10% HCC13; a third series was incubated at 45 C.; and a fourth was left untreated and incubated with the first and second series at 37 C. The object of this procedure was to determine whether after the maximum growth period was reached

and the cultures were inactivated by chemical or heat, with the enzymes still capable of activity, hemolytic substances were being produced. Each day tests were made for the presence of hemolysins. After the first day, guinea-pig serum and a living (24-hour) culture in 1 c c quantities were added to the 45 C. specimen. This was to supply complement, if it were needed, and any other vital substances necessary for hemolysis that a growing culture might possess. The results are appended in table 2, which shows: 1. No hemolysin was formed in cultures subjected to antiseptics or heat. 2. Complement does not appear necessary for hemolysis. 3. A living culture produces hemolysis per se, and is

TABLE 2
SHOWING EFFECT OF HEAT AND CHEMICAL AGENTS ON PRODUCTION OF HEMOLYTIC
SUBSTANCE

	Days									
	1	2	3	4	5	6	7	8	9	10
Strain A5 (From Air)										
Phenol.....	—	—	—	—	—	—	—	—	—	—
HCCls.....	—	—	—	—	—	—	—	—	—	—
45 C.	—	—	—	—	—	—	—	—	—	—
45 C. + complement.....	*	—	—	—	—	—	—	—	—	—
45 C. + 24 hour culture.....	*	+	+	+	+	+	+	+	*	*
Untreated.....	+	+	+	+	+	+	+	±	—	—
Phenol.....	—	—	—	—	—	—	—	—	—	—
HCCls.....	—	—	—	—	—	—	—	—	—	—
45 C.	—	—	—	—	—	—	—	—	—	—
45 C. + complement.....	*	+	—	—	—	—	—	—	—	—
45 C. + 24 hour culture.....	*	+	+	+	+	+	+	+	*	*
Untreated.....	+	+	+	+	+	+	+	±	—	—
Phenol.....	—	—	—	—	—	—	—	—	—	—
HCCls.....	—	—	—	—	—	—	—	—	—	—
Complement.....	*	—	—	—	—	—	—	—	—	—
Strain A5.....	*	+	+	+	+	+	+	+	*	*
Strain T9.....	*	+	+	+	+	+	+	+	*	*
Salt.....	—	—	—	—	—	—	—	—	—	—

* = no test conducted. Day 1 is 1st day under treatment, but 6th day of age of culture.

consequently worthless in such an examination. These results do not show that hemolysin is of an autolytic nature; neither do they show that it is not of an autolytic nature. The conclusion to be drawn is that in the case of heat, the hemolysin being thermolabile, is possibly dissipated; while in the case of the antiseptics, the hemolysin is so closely associated with the bacterial cell that destruction of the latter means lack of manifestation from the former. This falls somewhat in line with the work of Gordon on meningococci, showing that hemolysins are endocellular and are liberated on autolysis of the bacterial cells.

Effect on Hemolytic Activity of Successive Transplantation in Blood-Free Medium.—The object of the next experiment was to

determine whether a hemolytic strain of staphylococcus is always hemolytic. No definite references to the loss of this haemolytic manifestation could be found in the literature. Transplants were made daily into peptone broth, and at the end of each week blood-agar plates were streaked to show whether the cultures were still hemolytic. After the second week, since the plates were readily hemolyzed, the cultures were transplanted every other day, and after the first month every week. The reason for this change of procedure was the assumption that by daily transplantations the cultures were kept very active and that it would be more difficult, if possible, to suppress so vital a quality.

This experiment was continued for more than four months, and at the time of writing the cultures were still hemolytic. On some occasions there appeared to be retardation in hemolysins, and then the following week the cultures were as actively hemolytic as originally. Since the retardation was neither progressive nor continuous, it is reasonable to assume that it was probably due to differences in the blood used for the work. Normal horse blood, which was used for the blood-agar plates in this experiment; has been shown by Neisser and Wechsberg² to possess small quantities of antihemolysin. This normal quantity, however, may have been sufficient to delay hemolysis. It would seem, therefore, that hemolytic cultures tend to remain hemolytic.

Effect on Nonhemolytic Strains of Successive Transplantations in Blood Medium.—In this case the point at hand was to determine whether nonhemolytic cultures could be made hemolytic by adaptation to blood medium. If nonhemolytic cultures can be made to lake blood, it may be said that any strain of staphylococcus is hemolytic, adding provisionally that continual adaptation to a blood-free habitat ultimately suppresses its hemolytic activity and keeps it in abeyance; but readaptation to a blood-containing medium will restore the suppressed activity. Table 3 shows that after a period of 7 months, certain strains regained their hemolytic ability. It may be that this power was recovered at an earlier period, but tests were not definitely made until the stated lapse of time.

It should be added here, in view of a wealth of work in a hospital laboratory, that we think every strain of staphylococcus is definitely hemolytic. The strains will vary in degree of hemolysis, and in rapidity of hemolysis, but if sufficient time is given, all strains will show hemolysis. When the strains under study were isolated, a period of 6 days was given to determine hemolysis on blood plates, and it is now apparent

that the 6 days were not sufficient. Other cultures not included in this survey did show hemolysis after the arbitrarily chosen time, and attempts to collect nonhemolytic strains after 10 and in rarer cases 12 days, have failed. So that it seems by virtue of this evidence that the strains we originally labeled nonhemolytic were in reality hemolytic, and that their hemolytic character was very much suppressed. It would seem, therefore, that it can be definitely stated that cultures which did not show hemolysis within 6 days were able to give definite signs of hemolysis on blood plates within 24 hours and complete hemolysis within 48 hours.

Since the completion of this experiment every strain of staphylococcus isolated (whether a contaminant or a pathogen) was held for study. The number of days required to show beginning hemolysis was recorded. These results are tabulated in table 4. It will be seen that every strain shows hemolysis, but that the factor of time plays an

TABLE 3
DEVELOPMENT OF HEMOLYSIS BY NONHEMOLYTIC ? STRAINS

	April, 1921	November, 1921		April, 1921	November, 1921
A 1	No hemolysis	Hemolysis	P 5	No hemolysis	Hemolysis
A 2	Hemolysis	Hemolysis	S 2	Hemolysis	Hemolysis
A 3	Hemolysis	Hemolysis	T 1	No hemolysis	Hemolysis
A 5	Hemolysis	Hemolysis	T 2	Hemolysis	Hemolysis
F 1	No hemolysis	Hemolysis	T 3	Hemolysis	Hemolysis
H 2	No hemolysis	Hemolysis	T 5	Hemolysis	Hemolysis
P 1	Hemolysis	Hemolysis	T 6	Hemolysis	Hemolysis
P 2	Hemolysis	Hemolysis	T 8	No hemolysis	Hemolysis
P 3	Hemolysis	Hemolysis	T 9	Hemolysis	Hemolysis
P 4	Hemolysis	Hemolysis	X	No hemolysis	Hemolysis

important part. Thus it is seen that in a general way aureus strains show hemolysis earlier, and that virulent strains also show hemolysis earlier than the saprophytic; but the point is clear that white and aureus strains, saprophytic and parasitic alike, become hemolytic. In the case of nipples, for example: These are supposedly sterilized and sent to the laboratory to be tested for sterility so that it is logical to assume that any growth is apt to be contamination. Yet the 9 white strains are as rapidly hemolytic as the 18 orange strains isolated from pus.

Effect of Carbohydrates on Hemolysis.—It has been shown by Ruediger,¹⁰ Lyall,¹¹ Davis,¹² Sekiguchi,¹³ Stevens and Koser¹⁴ and

¹⁰ Ibid., 1906, 3, p. 663.

¹¹ Jour. Med. Res., 1914, 30, p. 515.

¹² Davis: Jour. Infect. Dis., 1917, 21, p. 308.

¹³ Ibid., 1917, 21, p. 475.

¹⁴ Jour. Exper. Med., 1919, 30, p. 539.

others, that carbohydrates prevent hemolysis by streptococcus, and it was problematic just what their effect on staphylococcus hemolysis would be. Two experiments were carried out to determine this point. In the one case, cultures were planted into 10% serum broth plus 1% dextrose. After 9 days tests were made for hemolysis and H-ion concentration read—to assure ourselves that an acidity would not interfere with the test. The results were: for 10% serum broth, dextrose 1%, strain A5 gave P_H 6.4, hemolysis and strain T9, P_H 7.6 and hemolysis.

Incidentally both these strains were streaked on lactose-blood-agar plates, and in both cases hemolysis was produced within 24 hours. In the second case, cultures were planted into peptone broth plus 1% dextrose. The test for hemolysis was positive after 24 hours, but the

TABLE 4
HEMOLYTIC ACTIVITY OF CONSECUTIVE CULTURES

Source	Pigment	No. of Cultures	Average Time for Hemolysis
Air.....	White	8	6-7 days
	Yellow	2	1-2 days
Sputum.....	White	1	3 days
	Aureus	2	1 day
Skin.....	White	4	4 days
Throat.....	White	4	1 day
	Yellow	1	1 day
Tonsil.....	White	1	1 day
	Aureus	1	1 day
Nipple.....	White	9	1-2 days
	Aureus	2	1 day
	Yellow	2	1 day
Feces.....	White	2	3 days
	Yellow	1	2 days
Pus.....	Yellow	3	2 days
	Orange	18	1-2 days
Neeropsy.....	Aureus	3	1-2 days
	Yellow	1	1 day
Water.....	White	2	4 days
Contamination (source unknown....)	White	3	7 days

hemolysis was not typical, showing a browning similar to acid hematin formation. Consequently the P_H value was determined and found to be 4.4. The reaction was adjusted to neutrality and hemolysis no longer took place. Approaching the question from another tangent, sterile salt solution adjusted to a reaction of P_H 4.4 caused the same type of hemolysis.

It would seem from these experiments that carbohydrates do not influence hemolysis as produced by staphylococcus. Regarding the acidity produced in the peptone broth and not in the serum broth, it is easily conceivable that the buffer qualities of the serum in the latter obscure the acid formed by fermentation of dextrose.

Effect of Heat on Hemolysis.—Neisser and Wechsberg² found that heating the staphylococcus "hemolysin" for 20 minutes at 56 C. would completely inactivate it.

In determining the effect of heat on the hemolytic action, the supernatant fluids of centrifuged 9-day cultures were heated at 56 C. for 30 minutes, and it was found that the hemolysin of staphylococcus is a thermolabile substance, which can be destroyed by heating in this way.

DISCUSSION

Previous investigators of the hemolytic activity of staphylococcus were concerned with observations of the hemolytic activity per se. Aside from some speculations as to its relation to pigment, virulence and agglutination, no attempt was made to arrive at its causation. The point under study here was concentrated on the cause of the hemolytic activity, and the period of its development was only a coincidental observation, since this phase of it was already sufficiently elaborated by previous investigators.

Our results point to a process of proteolysis—perhaps associated with autolysis—as the cause of hemolysis. This is not a new conception—it has been shown to be the fundamental of meningococcus hemolysis and were experiments performed to establish the point of possibly *B. proteus*, *B. coli*, etc. Although we have been unable to demonstrate irrevocably that autolysis is the specific cause, it is very significant that the period of maximum growth first appears, then the period of maximum amino acidity, and, finally, the period of maximum hemolysis. Such a sequence of evidence can point only to autolysis.

It must be for this reason that we have been unable to suppress the hemolytic activity of our hemolytic strains. If hemolysis is due to so important a function as protein-splitting, the factor involved is too vital to be eradicated by continued growth in blood-free mediums. Conversely, it is no wonder that slowly hemolytic cultures will increase in rapidity of hemolysis by continued adaptation to an environment where protein utilization becomes more pronounced.

Nor is it phenomenal that sugar should not inhibit hemolysis in such a case. Kendall and Walker's¹⁵ conception that the presence of glucose has a protein sparing effect and consequently retards production of proteolytic enzymes can be accepted only provided the hydrogen-ion concentration of the medium increases within suitable limits. For as

¹⁵ Jour. Infect. Dis., 1915, 17, p. 442.

Berman and Rettger¹⁶ pointed out, in tests in which buffers are employed proteolytic enzymes appear as soon in sugar mediums as in plain broth. And in serum broth, the buffer qualities of serum cannot be denied.

II. RELATIONSHIP OF HEMOLYTIC ACTIVITY TO OTHER METABOLIC ACTIVITIES

This part of the investigation concerns itself with a study of the biochemical reactions of the staphylococci, particularly as possible relations to hemolysis. Although, as the evidence submitted will show, hemolysis appears to be a separate entity from the biochemical reactions pursued, some new points of interest have been added to the literature of the hemolytic staphylococci.

CHROMOGENESIS

Except in a general way, a distinction of the chromogenic varieties of the staphylococci is an insignificant one. The pigment produced by bacteria is influenced to a greater or less extent by the medium employed for its production, and can be greatly modified by selection or by previous environment. Loeffler's serum medium, for example, without affecting the inherent power of chromogenesis always accentuates the depth of color produced by staphylococci. Pigment will vary with the amount of oxygen, the amount of moisture available, and the age of the culture.

So Neisser and Lipstein¹⁷ offer the hypothesis that white cocci were originally orange cocci which have lost their chromogenic power. Rodet and Courmont¹⁸ published the observation of the transformation of a white staphylococcus to an aureus and subsequently to a white again. Lubinski¹⁹ showed that the orange forms lost their pigment when grown anaerobically; in some cases the recovery was delayed and in other cases the loss was permanent. Kolle and Otto²⁰ stated that chromogenic cocci lose their chromogenesis by heating to 85 C., by prolonged cultivation on artificial mediums, and by repeated animal passage. Winslow and Rogers²¹ showed that a temperature of 50-55 C. may cause a loss in chromogenesis.

Neisser and Wechsberg showed that strains of both *Staphylococcus albus* and *Staphylococcus aureus* would produce hemolysins. This was later corroborated by both Kutscher and Konrich²² and Koch.²³ Noguchi²⁴ and Rosen-

¹⁶ Jour. Bact., 1918, 3, p. 389.

¹⁷ Handbuch. d. pathog. Mikroorganismen, 1914, 3, p. 105.

¹⁸ Compt. rend. Acad. d. sc., 1890, 9, p. 186.

¹⁹ Centrallbl. f. Bakteriolog., 1894, 16, p. 769.

²⁰ Ztschr. f. Hyg. u. Infektionskr., 1902, 41, p. 369.

²¹ Jour. Infect. Dis., 1906, 3, p. 485.

²² Zetschr. f. Hyg. u. Infektionskr., 1904, 48, p. 249.

²³ Ibid., 1907, 58, p. 287.

²⁴ Arch. f. klin. Chir., 1911, 96, p. 696.

bach²⁵ show a relation between virulence and pigmented cocci, while Passet²⁶ and Fisher and Levy²⁷ show that the lightly colored or colorless forms are most often associated with disease processes.

EXPERIMENTS

In determining chromogenesis the technic employed was that suggested by Winslow and Winslow.²⁸ Cultures were grown on agar slants at 20 C. for 2 weeks. A portion of the growth was spread over white roughened paper, with a platinum loop and allowed to dry in air. The hue and tint were matched against the colors of the frontispiece of their book (table 5).

TABLE 5
SOURCES AND CHROMOGENESIS OF THE STRAINS STUDIED

A 1—From the air.....	Lemon yellow I
A 2—From the air.....	Medium cadmium yellow IV
A 3—From the air.....	Cadmium orange III
A 5—From the air.....	Medium cadmium yellow IV
F 1—From feces.....	White
H 2—From heart's blood at necropsy.....	Medium cadmium yellow V
P 1—From pus from spine.....	Cadmium orange IV
P 2—From pus from carbuncle.....	Lemon yellow I
P 3—From pus from acne.....	Cadmium orange IV
P 4—From pus from extracted tonsil.....	Cadmium orange IV
P 5—From pus (unclassified).....	Cadmium orange IV
S 2—From skin.....	White
T 1—From throat.....	Orange yellow III
T 2—From throat.....	Medium cadmium yellow V
T 3—From throat.....	Medium cadmium yellow VI
T 5—From throat.....	Lemon yellow II
T 6—From throat.....	Lemon yellow III
T 8—From throat.....	White
T 9—From throat.....	Orange yellow V
X—From blood culture (case ferunculous).....	Orange yellow III
C15—From throat.....	White
C16—From throat.....	White
C18—From throat.....	White
J 1—From pus.....	White
L 1—From pus.....	Cadmium orange IV

It will be seen at a glance that there is no relationship between pigment and hemolysis. The cultures are all hemolytic, and yet they vary from a white to a rich golden brown. This is scarcely surprising. The literature shows that pigment production may be varied, and while the hemolytic activity seems to be fixed, it could hardly be expected that the two functions would be related.

ACID PRODUCTION IN THE PEPTONE MEDIUM OF CLARK AND LUBS

Preparatory to the carbohydrate metabolism studies of staphylococci, this experiment was made to determine in a general way any relation-

²⁵ Dent. med. Wchnschr., 1884, 6, p. 31.

²⁶ Passet: Fortschr. d. Med., 1885, 33, p. 33.

²⁷ Dent. Ztschr. f. Chir., 1893, 36, p. 94.

²⁸ Systematic Relationships of the Coccocese, 1908.

ship between hemolysis and acid production. In view of the methyl red test of differentiation of *B. coli* and *B. aerogenes* by this medium, it seemed at the time that it might possess some value in this work. The peptone medium contained 0.5% K_2HPO_4 , 0.5 peptone (Difco), and 0.5% dextrose, and was adjusted to P_H 7.4.

Table 6 shows the H-ion readings of the different cultures from time to time as specified. With the exception of A1, all strains reach an end-point of P_H 4.2-4.6 within 96 hours. Although there seem to be differences in the earlier readings, there is no line of demarcation between the acid production of the cultures. These differences are probably explainable on differences in numbers inoculated, periods of lag, etc.

TABLE 6
ACID PRODUCTION IN CLARK AND LUBS MEDIUM *

	8 Hours	12 Hours	16 Hours	20 Hours	24 Hours	48 Hours	72 Hours	96 Hours
A 1.....	7.6	7.6	7.4	7.0	6.9	6.9	6.9	6.9
A 2.....	6.4	4.6	4.6	4.6	4.6	4.4	4.4	4.4
A 3.....	6.2	4.6	4.4	4.4	4.4	4.4	4.4	4.4
A 5.....	4.6	4.4	4.2	4.2	4.2	4.2	4.2	4.2
F 1.....	6.0	5.8	5.8	5.0	4.6	4.6	4.6	4.6
H 2.....	5.0	4.6	4.4	4.4	4.4	4.4	4.4	4.4
P 1.....	6.1	4.9	4.6	4.6	4.6	4.6	4.6	4.6
P 2.....	6.0	4.6	4.6	4.6	4.6	4.6	4.6	4.6
P 3.....	6.8	4.6	4.6	4.6	4.6	4.6	4.6	4.6
P 4.....	5.0	5.0	4.8	4.6	4.6	4.6	4.6	4.6
P 5.....	6.6	5.0	4.6	4.4	4.4	4.4	4.4	4.4
S 2.....	7.6	5.8	5.0	5.0	4.6	4.6	4.6	4.6
T 1.....	5.0	5.0	4.8	4.8	4.6	4.6	4.6	4.6
T 2.....	5.4	4.8	4.8	4.6	4.6	4.6	4.6	4.6
T 3.....	5.0	4.9	4.9	4.9	4.6	4.4	4.4	4.4
T 5.....	5.0	4.8	4.6	4.4	4.4	4.4	4.4	4.4
T 6.....	7.6	6.6	6.0	5.4	4.6	4.6	4.6	4.6
T 8.....	5.5	5.0	5.0	4.8	4.8	4.8	4.6	4.6
T 9.....	7.4	5.6	5.0	5.0	4.6	4.6	4.6	4.6
X.....	6.4	4.4	4.4	4.4	4.4	4.4	4.4	4.4
Control.....	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2

* Figures represent values of H-ion concentration.

At this point, the question arose as to what determined the acid end-point of the cultures. To approach an answer, 2 experiments were planned: (1) Cultures were grown in the same medium with the reaction adjusted to P_H 4.4; (2) cultures which had already reached an acidity of P_H 4.4 were killed by heating at 56 C. for 30 minutes and inoculated with a 24-hour culture.

In both these cases, the H-ion concentration was increased to 4.2 and 4 after 24 hours. It might be of interest to quote here the work of Hall and Frazer²⁰ who found that staphylococci could reach a H-ion concentration of 2.6—an end-point which exhibited no relation to saprophytic or pathogenic forms.

²⁰ Abstract, Lancet, 1921, 18, p. 912.

CARBOHYDRATE METABOLISM

In view of the diagnostic importance of the fermentative reaction of the colon-typhoid group, it was deemed advisable to devote considerable attention to this subject. Very little previous work has been done on the ability of the staphylococci to ferment carbohydrate mediums. Of course, it is common knowledge that they attack the more familiar sugars with the production of acid, but no gas. Gordon,³⁰ in reporting a classification study of the white cocci, gave the fermentation reactions on lactose, maltose, glycerol and mannitol. Dudgeon³¹ reported a comparative study of the aureus and albus cocci, studying among other things their acid production in 11 carbohydrate mediums; but none of his results were quantitative. Winslow and Winslow²⁸ studied glucose and lactose, and Kligler³² glucose, lactose and sucrose. More recently Winslow and his co-workers³³ made a quantitative study of the acid produced in 9 different sugars. They found more than half the strains studied fermented glucose, maltose and sucrose; about half fermented lactose; 5 strains fermented salicin, 1 strain each fermented inulin and raffinose, and no strains fermented dulcitol and mannitol.

In our study, we have employed 17 carbohydrates in all-dextrose, galactose, levulose, sucrose, lactose, maltose, raffinose, arabinose, inulin, dextrin, salicin, adonitol, mannitol, sorbitol, dulcitol, glycerol and starch. Twenty-four hour cultures were inoculated into 1% peptone broth plus 1% of the carbohydrate designated. The cultures were incubated at 37 C. for one week, and the P_H value determined by matching the tubes against the Clark and Lubs⁶ standards. In table 7 the P_H values alone are given, since gas was not formed in any case.

The table shows that the carbohydrates are either fermented or not; but in either case the reaction is uniform. There are slight differences in some of the mediums, but they are not important enough for classification; they indicate merely functional differences and as such are negligible.

To compress the table:

Carbohydrates Fermented	Not Fermented
Glucose	Starch
Galactose	Dulcitol
Levulose	Adonitol
Sucrose	Dextrin
Lactose	Inulin
Maltose	Arabinose
Salicin	Raffinose
Mannitol	
Sorbitol	
Glycerol	

³⁰ Quoted by Winslow and Winslow. Supplement to the 34th annual report of local gov't. bd. containing the report of the Med. officer for 1904-1905, p. 387.

³¹ Jour. Path. & Bacteriol., 1908, 12, p. 242.

³² Jour. Infect. Dis., 1913, 12, p. 432.

³³ Winslow, Rothberg and Parsons: Jour. Bacteriol., 1920, 5, p. 145.

The discrepancy in uniformity of fermentation between this study and that of Winslow and others is possibly due to the fact that they included in their survey strains of *Staph. epidermidis*, *ureae*, *candidus*, *tetragenus*, *candicans*, *aureus* and *aurianticus*, thereby making a survey of many less active organisms than those employed in our study.

PROTEIN METABOLISM

Decomposition of Peptone to Amino Acids.—As a rule, the only accessible figures of amino acid formation of staphylococci occur scattered through bacteriologic literature where the question at hand was primarily a study of the nitrogen metabolism of several species and

TABLE 7
FERMENTATION OF CARBOHYDRATES

	Arabinose	Dextrose	Galactose	Levulose	Sucrose	Lactose	Maltose	Inulin	Dextrin	Adonite	Salicin	Mannitol	Sorbitol	Dulcitol	Glycerol	Starch	Raffinose
A 1	7.4	6.2	5.9	7.0	6.2	6.2	6.2	7.8	7.1	7.6	6.0	6.2	6.0	7.7	6.0	8.0	7.8
A 2	7.3	4.4	4.7	5.0	5.0	4.8	7.5	7.1	7.6	6.0	4.6	4.7	7.7	6.0	8.0	7.7	7.7
A 3	7.2	4.6	4.7	5.0	4.8	5.6	4.8	7.5	7.1	7.6	6.0	5.0	4.7	7.7	6.0	8.0	7.6
A 5	7.2	4.4	4.6	5.0	4.8	5.0	4.8	7.5	7.6	7.6	5.8	5.0	4.7	7.7	6.0	8.0	7.8
F 1	7.0	4.6	4.6	5.0	4.9	5.0	4.8	7.5	7.8	7.6	6.0	5.4	4.7	7.7	6.0	8.0	7.7
H 2	7.3	4.4	4.7	5.0	4.8	5.0	4.8	7.5	7.1	7.6	6.0	5.2	4.7	7.8	6.0	8.0	7.7
P 1	7.3	4.4	4.7	5.0	4.6	5.1	4.6	7.5	7.1	7.6	6.0	5.0	4.7	7.7	6.0	8.0	7.7
P 2	7.2	4.4	6.0	5.2	4.8	5.0	4.8	7.5	7.1	7.5	5.4	5.0	4.7	7.7	6.0	8.0	7.8
P 3	7.3	4.5	4.7	5.2	4.8	5.0	6.0	7.5	7.1	7.6	6.0	4.6	4.7	7.7	5.0	8.0	7.8
P 4	7.2	4.4	4.7	5.0	4.7	5.1	5.4	7.5	7.2	7.6	6.0	4.6	4.7	7.6	5.0	8.0	7.8
P 5	7.3	4.9	5.1	5.2	4.9	5.1	5.6	7.5	7.1	7.6	6.2	5.4	4.7	7.7	6.0	8.0	7.5
S 2	7.3	4.6	4.7	5.2	4.8	5.4	4.8	7.5	7.1	7.6	6.0	4.6	4.7	7.7	6.0	8.0	7.7
T 1	7.3	4.4	4.7	5.0	4.8	5.0	4.8	7.5	7.1	7.8	6.0	5.3	4.7	7.6	5.2	8.0	7.7
T 2	7.3	4.4	4.7	5.0	4.6	5.1	4.8	7.5	7.2	7.6	6.0	5.2	4.7	7.7	6.0	8.0	7.7
T 3	7.3	4.4	4.7	5.0	5.0	5.0	4.2	7.6	7.2	7.6	6.2	4.6	4.7	7.7	5.0	8.0	7.7
T 5	7.2	4.4	4.7	5.0	4.8	5.1	4.8	7.3	7.2	7.6	6.0	4.6	4.7	7.7	6.0	8.0	7.7
T 6	7.3	4.6	4.7	5.2	4.8	5.0	4.8	7.5	7.1	7.6	6.0	4.6	4.7	7.7	6.0	8.0	7.7
T 8	7.3	4.4	4.8	5.0	4.8	5.6	4.8	7.5	7.0	7.4	6.0	4.6	4.7	7.6	5.0	8.0	7.6
T 9	7.3	4.6	4.9	5.0	4.8	5.2	4.8	7.5	7.1	7.4	6.0	5.2	4.7	7.7	6.0	8.0	7.9
X	7.3	4.4	4.7	5.2	5.0	5.0	4.8	7.5	7.1	7.4	5.4	5.4	4.7	7.0	5.0	8.0	7.7
Control	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.2

one or two strains of staphylococci were fortuitously included. Our object here was to give a definite conception of the amino acid digestion of peptone, and incidentally to use such an expedient for a classification, if possible.

Rosenthal and Patai³⁴ found that the curve of amino acid production by staphylococcus underwent an initial sharp rise within 24 hours, and this was followed by a more gradual rate of increase until the fifth and sixth day. Also virulent organisms produced more amino acid than the avirulent ones. Their determinations were made by the Sørensen method. The work of Berman and Rettger³⁵ shows that at the end of 1 week 3 strains of *aureus* reached an

³⁴ Centralbl. f. Bakteriöl., I, O., 1914, 73, p. 406.

³⁵ Jour. Bacteriol., 1918, 3, p. 367.

amino acid figure equivalent to 47 c.c. of 20/NaOH, and one strain of albus, a figure of 52 c.c. of 20/NaOH. They also used the Sørensen method.

Benton³⁶ recently observed that in 1.5% peptone broth, staphylococcus shows a decrease in amino acidity until the 5th day, with a following rise until the 7th day; in 2% peptone broth, the decrease continues until the 3rd day with a gradual increase until the 9th day; in pure ascitic fluid, after a 1 day decrease, there is a rise until the 4th day. She used the Van Slyke method for amino acid determination.

In our own experiment, a 2% Difco peptone extract broth was employed. The tubes were inoculated with a 24-hour growth and at the end of each day the amino acidity was determined by the Sørensen method (table 10).

TABLE 8
AMINO ACID DECOMPOSITION OF PEPTONE *

	1st Day		2d Day		3d Day		4th Day		5th Day	
	P _H	A. A.	P _H	A. A.	P _H	A. A.	P _H	A. A.	P _H	A. A.
A 1.....	7.5	40.0	7.5	48.0	7.5	72.0	7.5	100.0	7.5	44.0
A 2.....	7.5	60.0	7.7	48.0	7.6	84.0	7.7	112.0	7.9	72.0
A 3.....	7.5	68.0	7.7	68.0	7.7	88.0	7.7	116.0	7.9	72.0
A 5.....	7.5	80.0	7.5	72.0	7.6	92.0	7.6	120.3	8.0	76.0
F 1.....	7.5	68.0	7.5	72.0	7.8	96.0	7.8	120.0	8.0	60.0
H 2.....	7.5	68.0	7.5	96.0	7.7	112.0	7.8	116.0	8.0	72.0
P 1.....	7.8	56.0	7.9	56.0	7.8	96.0	7.8	116.0	8.0	72.0
P 2.....	7.7	56.0	7.7	80.0	7.7	96.0	7.7	116.0	8.0	72.0
P 3.....	7.5	56.0	7.9	84.0	8.0	96.0	7.7	116.0	7.7	64.0
P 4.....	7.5	56.0	7.9	72.0	7.9	100.0	7.7	92.0	7.7	68.0
P 5.....	7.3	52.0	7.4	68.0	7.5	76.0	7.5	76.0	8.0	64.0
S 2.....	7.7	48.0	7.8	76.0	7.8	72.0	7.8	92.0	8.0	80.0
T 1.....	7.8	80.0	7.9	116.0	7.9	116.8	8.0	140.0	8.0	68.0
T 2.....	7.5	56.0	7.9	84.0	7.9	96.0	7.9	80.0	8.0	68.0
T 3.....	7.5	56.0	7.9	72.0	7.9	96.0	7.9	96.0	8.0	68.0
T 5.....	7.5	56.0	7.6	72.0	7.8	88.0	7.5	80.0	8.0	40.0
T 6.....	7.5	56.0	7.8	68.0	7.7	92.0	7.7	72.0	7.9	60.0
T 8.....	7.7	56.0	7.9	116.0	8.0	116.0	7.7	132.0	7.7	104.0
T 9.....	7.6	44.0	7.7	80.0	7.8	100.0	7.8	72.0	7.9	64.0
X.....	7.8	52.0	7.8	72.0	7.8	92.0	7.8	116.0	8.0	64.0
Control.....	7.0	48.0	7.0	48.0	7.0	48.0	7.0	48.0	7.0	48.0

* In P_H column, P_H readings are given. In A. A. column, figures represent number of c.c. of 20 N NaOH required to neutraliz 100 c.c. of culture.

The significant features brought out are that at the end of the first day there is either a slight increase or decrease in amino acid, then a gradual rise to the 4th day, with a falling off on the 5th day. At the time, we assumed that a maximum had been reached on the 4th day. This checks fairly well with the results of Rosenthal and Patai, but brings our maximum a bit sooner than was the case in Benton's work.

These results differ materially from those obtained with serum broth, but the mediums were of course different. It would seem that in serum broth the amino acids are simultaneously formed and utilized,

and thus the figures are kept low; whereas in peptone broth, the amino-acidity figures increase rapidly due to the greater amounts of peptone present.

Production of Ammonia.—This test was performed for a double purpose: In the first place, it was interesting to determine what happened to the amino acid formed, and in the second place, to determine whether any differentiation could be made on this basis. The amount of ammonia formed was measured daily for 5 days after incubating at 37 C. The medium employed was composed of 1% peptone and 0.05% K_2HPO_4 . The tubes were sealed with paraffin to prevent the escape of ammonia. The determination was made with Nessler reagent, and the cultures were matched against a known standard by

TABLE 9
AMMONIA FORMATION

	2d Day	3d Day	4th Day	5th Day
A 1.....	6.4	9.72	11.60	21.84
H 2.....	13.80	19.20	20.40	14.64
H 3.....	12.00	17.76	20.50	16.44
H 5.....	12.00	15.36	23.40	16.92
F 1.....	9.60	20.40	24.00	26.04
H 2.....	14.16	21.84	27.04	21.00
P 1.....	12.84	14.60	16.80	16.78
P 2.....	12.60	14.60	19.20	21.84
P 3.....	9.60	21.60	30.00	21.84
P 4.....	17.16	20.40	30.00	32.88
P 5.....	9.60	14.16	15.48	30.00
S 2.....	12.96	14.40	28.20	24.00
T 1.....	24.00	27.00	48.00	48.00
T 2.....	13.32	30.00	32.88	21.36
T 3.....	16.78	23.20	24.00	14.64
T 5.....	14.76	21.60	32.80	28.20
T 6.....	14.16	17.96	32.80	28.56
T 8.....	32.80	38.40	64.80	57.12
T 9.....	14.60	16.68	32.80	14.60
X.....	19.20	23.40	24.00	16.44
Control.....	1.98	1.98	1.98	1.98

Figures represent mg. of NH_3 as nitrogen per 100 c.c. of culture.

means of the Dubosq colorimeter. From table 9 it will be seen (1) that all the cultures produce ammonia, and (2) that amino-acidity and ammonia formation are simultaneous processes. Winslow, Rothberg and Parsons report positive ammonia formation in all but 11 strains out of 180 studied.

Reduction of Nitrates.—Gordon³⁰ and Winslow,²⁸ Rothberg and Parsons³³ found that nitrate reduction by staphylococci was a more or less general character. Winslow and Winslow²⁸ reported only 21% aureus and 13% albus reducers. Kligler's study showed 7 out of 11 aureus and only 1 out of 12 albus reduced nitrates. The more recent work of Winslow, Rothberg and Parsons³³ had the advantage of better technic and should have the greatest weight.

In making our determination, the medium contained 1% peptone, 0.5% K_2HPO_4 and 1% KNO_3 . The cultures were incubated for one week at 37 C., and the presence of nitrates was determined by the sulphanilic acid— α -naphthalamine method. All the strains except A were able to reduce nitrates.

Formation of Indol.—In a survey of the literature of indol production by staphylococci, 3 references have been found of a positive nature. Emmerling³⁸ described the production of indol after 14 days' cultivation under anaerobic conditions on an egg white medium. Tissier and Martelly³⁹ reported positive indol by a culture of *Staphylococcus albus* isolated from meat, and cultivated in a fibrin medium. Distaso⁴⁰ isolated an atypical staphylococcus which was an obligate anaerobe and showed inability to attack any sugar, but which was capable of forming indol. The results of the first two are questionable on account of the technic employed, while the third case is concerned with an atypical organism. On the other hand, negative indol production is reported by Buard,⁴¹ Seltzer,⁴² Dobrowski,⁴³ Distaso,⁴⁴ Zipfel,⁴⁵ Herzfeld and Klinger,⁴⁶ Winslow, Rothberg, and Parsons,³³ and Bayne-Jones and Zimmiger.⁴⁷

Our tests were made by cultivating in a medium of 1% peptone and 0.5% K_2HPO_4 at 37 C. Tests for the presence of indol were made on the first, third, fifth, seventh and tenth day after incubation by the para-dimethyl-amido-benzald-benzaldehyde method. All tests were negative.

Action on Milk.—Table 10 gives the reaction of each strain in litmus milk. It will be seen that after 10 days' incubation at 37 C., one strain shows no apparent change in reaction, 11 strains show acid production, and 8 strains show acid with coagulation and liquefaction. The P_H values in lactose broth has been placed alongside the milk reactions. As was expected, the reaction coincides.

Liquefaction of Gelatin.—In determining gelatin liquefaction, an effort was made toward a quantitative study. The technic employed was to inoculate gelatin tubes with 0.1 c c of a 24-hour broth culture (diluted if necessary to insure an even turbidity). The amount of gelatin liquefied was measured by determining the number of c c from a mark drawn at the original level of the gelatin to the level of the

³⁸ Berlin der Deutsch. Chem. Gessellsch., 1896, 29, p. 2721.

³⁹ Ann. de l'Inst. Pasteur., 1910, 24, p. 865.

⁴⁰ Centralbl. f. Bakteriöl., I, O., 1912, 62, p. 433.

⁴¹ Compt. rend. Soc. de biol., 1908, 65, p. 158.

⁴² Centralbl. f. Bakteriöl., I, O., 1909, 51, p. 465.

⁴³ Ann. de l'Inst. Pasteur., 1910, 24, p. 595.

⁴⁴ Centralbl. f. Bakteriöl., I, O., 1911, 59, p. 102

⁴⁵ Ibid., 1913, 67, p. 572.

⁴⁶ Ibid., 1915, 76, p. 1.

⁴⁷ Bull. Johns Hopkins Hosp., 1921, 32, p. 299.

nonliquefied gelatin. The cultures were incubated at 20 C. for 21 days, unless the gelatin was entirely liquefied before that time, when the liquefaction was estimated.

TABLE 10
SHOWING ACTION ON MILK

	1 Day	3 Days	5 Days	7 Days	10 Days	Lactose
A 1.....	No change	No change	No change	No change	No change	6.2
A 2.....	Acid	Acid	Acid	Acid	Acid	5.0
A 3.....	Acid	Coagulation	Coagulation	Liquefaction	5.6
A 5.....	Acid	Coagulation	Coagulation	Liquefaction	5.0
F 1.....	Acid	Coagulation	Coagulation	Liquefaction	5.0
H 2.....	Acid	Coagulation	Coagulation	Liquefaction	5.0
P 1.....	No change	Acid	Acid	Acid	Acid	5.1
P 2.....	No change	No change	Acid	Acid	Acid	5.0
P 3.....	No change	Acid	Acid	Coagulation	Liquefaction	5.0
P 4.....	Acid	Acid	Acid	Acid	Acid	5.1
P 5.....	Acid	Acid	Acid	Acid	Acid	5.1
S 2.....	No change	No change	No change	Acid	Acid	5.4
T 1.....	No change	Acid	Acid	Coagulation	Liquefaction	5.0
T 2.....	No change	No change	No change	Acid	Coagulation	5.1
T 3.....	No change	Acid	Acid	Acid	Acid	5.0
T 5.....	No change	No change	No change	No change	No change	5.1
T 6.....	No change	No change	Acid	Acid	Acid	5.0
T 8.....	Acid	Acid	Acid	Acid	Acid	5.6
T 9.....	Acid	Acid	Acid	Acid	Coagulation	5.2
X.....	Acid	Coagulation	Liquefaction	Acid	5.0

TABLE 11
LIQUEFACTION OF GELATIN

	Amount Liquefied	No. of Days
A 1.....	No liquefaction	21
A 2.....	5.2 c c	19
A 3.....	No liquefaction	21
A 5.....	7.1 c c	19
F 1.....	No liquefaction	21
H 2.....	No liquefaction	21
P 1.....	6.0 c c	19
P 2.....	1.0 c c	21
P 3.....	5.5 c c	18
P 4.....	No liquefaction	21
P 5.....	No liquefaction	21
S 2.....	3.0 c c	19
T 1.....	6.7 c c	17
T 2.....	5.5 c c	17
T 3.....	0.9 c c	21
T 5.....	3.8 c c	19
T 6.....	No liquefaction	21
T 8.....	No liquefaction	21
T 9.....	4.1 c c	21
X.....	2.0 c c	21
Control.....	No liquefaction	21

Manner of Liquefaction: Some time later the study of gelatin liquefaction was extended by an observation of the manner of liquefaction. Table 12 gives a graphic representation of the findings. Up to the 10th day, let us say, there is a pseudodifferentiation of 2 types: one type giving a saucer-shaped liquefaction and the second type giving

a cone-shaped liquefaction. After that the liquefaction proceeds uniformly in all the cultures by stratification. The difference, however, is so superficial that we would hardly suggest a classification on this characteristic.

One significant feature brought out by the tables is the ability of six cultures to attack gelatin—cultures which did not a year previously manifest this ability. This shows above all the variability of the organisms to be classified—a variability which emphasizes the fact that in order to classify staphylococci we must depend on more substantial characters than functional differences.

TABLE 12
RESULTS OF AGGLUTINATION *

	Serums				
	A 1	A 5	P 1	T 9	L 1
A 1.....	5120	0	20	40	20
A 2.....	0	2560	1280	0	1280
A 3.....	0	2560	1280	0	1280
A 5.....	0	2560	1280	0	1280
F 1.....	1280	20	20	40	20
H 2.....	80	320	320	0	1280
P 1.....	0	2560	1280	20	1280
P 2.....	1280	0	0	0	0
P 3.....	0	2560	640	2560	1280
P 4.....	80	640	320	0	640
P 5.....	0	320	640	40	1280
S 2.....	1280	0	0	0	0
T 1.....	0	2560	640	0	1280
T 2.....	640	2560	640	320	1280
T 3.....	0	2560	1280	0	1280
T 5.....	640	20	0	0	1280
T 6.....	0	2560	1280	40	0
T 8.....	640	0	0	20	40
T 9.....	640	2560	1280	640	1280
X.....	1280	2560	640	1280	1280
C 15.....	2560	160	80	80	20
C 16.....	640	0	20	20	20
C 18.....	640	0	20	20	20
J 1.....	80	1280	1280	80	1280
L 1.....	0	1280	1280	0	1280

* Figures represent the dilution at which agglutination was observed by naked eye reading. All controls were negative.

Reduction of Methylene Blue.—At the December, 1921, meeting of the Am. Assn. of Bacteriol., Avery reported his investigation of the use of methylene blue in differentiating hemolytic streptococci from human and dairy sources. He found that dairy strains—bovine and cheese—reduced methylene blue, but that the human strains did not. Because of these results, we tried reducing methylene blue by our staphylococcus strains. The technic of the test consisted in adding to a 24-hour broth culture varying dilutions of methylene blue, and covering with sterile paraffin. The cultures were reincubated for a second day,

when the results were read. It was found that all strains reduced or decolorized methylene blue at dilutions of 1:50,000 and 1:25,000; they showed partial decolorization at dilution of 1:10,000, except strains T8, C15 and J1, which were negative; and at a dilution of 1:1,000 all the strains were negative.

Hydrolysis of Sodium Hippurate.—Ayers and Rupp⁴⁸ found that hemolytic bovine streptococci could be differentiated from the human by the fact that the former could split sodium hippurate into glycoll and benzoic acid. We employed this test in our study to determine whether such a procedure would be of value in differentiating the staphylococci. The medium employed contained 1% peptone, 1% sodium hippurate 0.015% K_2HPO_4 , and the reaction was adjusted to P_H 7.2. The cultures were incubated at 37 C. for 7 days. At that time hydrolysis was determined by adding 0.5 c c of a 7 % $FeCl_3$ solution for every 2 c c of the culture medium; if hydrolysis had taken place an insoluble precipitate was formed, whereas the mixture became clear on standing several minutes if hydrolysis had not taken place. All the cultures were able to split sodium hippurate.

RELATION TO VIRULENCE

Although Neisser and Wechsberg² showed that aureus and albus strains alike are capable of hemolytic activity, their experiment seems to indicate that purely saprophytic forms never attain this faculty. This was corroborated later by Kutcher and Konrich²² and also by Koch.²³ Noguchi in presenting his results stated that hemolysis was proportional to the virulence of a strain, but the evidence he presents does not justify such a conclusion. Montegazza⁴⁰ was unable to demonstrate any definite relation between the intensity of an infection and the quantity of hemolysin produced.

In approaching an answer to the question of inter-relationship between virulence and hemolysis, two methods present themselves—either hemolytic strains will prove to be virulent, or nonhemolytic strains will be avirulent.

Following the first method, then, strains A5, P1, P3 and T9, all definitely hemolytic, were used. Twenty-four-hour broth cultures of each were inoculated in 1 c c quantities into the peritoneum of separate mice. No casualties occurring, the mice were killed, the peritoneums were washed with sterile saline, and the washings injected into a fresh mouse. Incidentally, cultures were made of the peritoneal exudate and heart blood as a check. This procedure was carried successively for

⁴⁸ Personal communication.

⁴⁰ *Biochem. Centralbl.* 1908, 8, p. 226.

3 days with 3 mice for each strain. After the third mouse, in no case was staphylococcus demonstrable by smear or culture from the peritoneum indicating complete overwhelming of the 4 strains. Cultures of the heart blood, which were made to test the invasive powers of the 4 strains, were negative each day. Here, if anything, the virulence of the strains should have increased by the animal passage, but instead the organisms decreased, the more resistant organisms lasting until the third passage. This would indicate that hemolysis is quite independent of virulence.

Later, in attempting to isolate a virulent strain, 3 different strains from pus were injected into rabbits. Two strains injected intravenously in amounts of 3 c c of a 24-hour broth culture caused no apparent effect. The third strain caused death in 0.5 c c amounts within 2 days, and 0.25 c c amounts within 1 week, presenting in this case typical staphylococcus lesions. This strain was used in our serologic work and designated as L1. The point of interest here, however, is that although the 3 strains were distinctly hemolytic, only 1 proved to be sufficiently virulent to kill a rabbit. The combined evidence of these 7 strains makes plausible the conclusion that hemolytic strains are not necessarily virulent.

The second method—that nonhemolytic strains would prove to be avirulent—was not tried. Nonhemolytic strains were not isolated during the course of the entire investigation. However, a glance at table 4 at this point will show that strains of an undoubtedly saprophytic character are hemolytic. In a general way, perhaps, the strains requiring the greatest time for hemolysis are probably the least virulent of any; but, on the other hand, the strains giving most rapid hemolysis may be saprophytic.

LEUKOCIDIN ACTIVITY

It was not the purpose in this experiment to make a study of the leukocidin produced by staphylococci. The subject has been well worked out. The purpose was rather to determine whether hemolytic activity bears any relation to leukocidin activity.

Van de Velde⁵⁰ first demonstrated leukocidin by filtration in 24-hour cultures. Later he and Denys⁵¹ showed that the leukocidin was not specific, but was a metabolic product which destroyed other tissue cells as well as leukocytes. Bail⁵² obtained a maximum production of leukocidin in 11 days. Neisser and Wechsberg² added considerably to the knowledge of staphylococcus leukocidin. Making use of the reduction of methylene blue by leukocytes, they found that leukocidin appears in filtrates after 4 days and reaches

⁵⁰ La Cellule, 1894, 10, p. 403.

⁵¹ Ibid., 1895, 11, p. 395.

⁵² Arch. f. Hyg., 1898, 32, p. 133.

a maximum after 1 week; that leukocidin was produced by white and orange strains; that the more virulent the strain the more leukocidin produced; that leukocidin was destroyed by heating at 56 C.; that normal horse and immune serum possesses antileukocidin; that leukocidin does not attack kidney cells.

In making our tests the same strains used for hemolytic activity were used. The cultures were inoculated each day into 10% serum broth for 16 days so that on the 17th day we had 16 cultures of each strain of from 1 day to 16 days old. The cultures were then centrifuged at high speed for 5 minutes, and 1 c c of the supernatant fluid was used for the test.

Leukocytes were obtained by injecting 8-10 c c of sterile aleuronat into the pleural cavity of guinea-pigs, and after 15 hours the animals were bled to death and the pleural exudate removed with a capillary pipet. An equal amount of 1.5% sterile sodium citrate was added to the cells to prevent coagulation.

The presence of leukocidin was determined by the methylene blue reduction test. The methylene blue consisted of 1 c c saturated solution of methylene blue, 20 c c absolute alcohol, and 29 c c distilled water. The minimum quantity of leukocytes to reduce methylene blue was first measured by using different amounts of leukocytes varying from 0.2 c c to 2 c c, the volume being made equal through the series with sterile salt solution. Two drops of methylene blue were added, and then the mixture was covered with a layer of sterile liquid paraffin to prevent reoxidation from the air. The tubes were incubated at 37 C. for 2 hours.

To twice the minimum quantity of the leukocytes found necessary to give reduction of methylene blue was added 1 c c of the supernatant centrifuged culture. The tubes were incubated at 37 C. for 1½ hours, when 2 drops of methylene blue and liquid paraffin were added. Incubation was continued for 2 hours more when the readings were made. In case of reduction, no leukocidins were present, since the leukocytes had not been injured.

It was found that leukocidin appeared on the 4th day and disappeared on the 8th day; and that only strains H2 and T9 produced leukocidins. Thus it is seen that H2, which did not show hemolysin production in broth cultures, produces most leukocidin, and A5, which produced most hemolysins, does not produce leukocidins. A1 is negative in both cases, while T9 is positive in both cases. However, strains A5 and H2 indicate distinctly that hemolytic and leukocidin activity are not dependent on each other.

Theoretically we would expect that the amount of leukocidin produced would bear a relation to the virulence of a strain, for the latter would depend to some extent on the former. Since virulence and hemolysis were found to be individual characters, it was hardly supposed that hemolysis would show any dependence on leukocidin production.

III. SEROLOGIC REACTIONS

As a final analysis, recourse was taken to differentiate the hemolytic staphylococci on a serologic basis. The impression is that although biochemical reactions may vary, serologic reactions if once positive will always remain positive. So, for example, the agglutinability of an organism may fluctuate quantitatively, but not qualitatively. For no other reason, then, this part of the work seemed to have the greatest promise. Both deviation of complement and agglutination tests were made, and the agglutination tests were supplemented by absorption tests.

In preparing immune serums, strains A1, A5, P1, T9 and L1 were employed. Salt suspensions were made from agar slants and rabbits were injected intravenously in 3 day periods, with 2 days between each period. Five-tenths c c of the suspensions was injected the first period, and this was increased 0.5 c c each period until a serum of sufficiently high titer was obtained.

COMPLEMENT FIXATION

The literature on the complement fixation of staphylococci is scant. The one reference available was that of Kolmer, Trist and Yagle⁵³ in relation to influenza. Using a *Staphylococcus aureus* antigen, they were unable to get fixation with either normal or influenza serum.

The antigens used in these experiments were suspensions of 24 cultures to which were added 0.1% formaldehyd. The preparation of the serum has already been described.

After going through the preliminaries of obtaining antigenic and complementary doses, the tests were made by incubating at 37 C. It was found that all 5 serums gave fixation with all of the antigens. There appears to be no qualitative differentiation of the different strains.

One more step was taken, and that was to determine whether there might be quantitative separation into groups by complement fixation. Four strains were picked at random, and the serum used in dilutions of 1:50, 1:100, 1:150. The results did not warrant extending the

⁵³ Jour. Infect. Dis., 1919, 24, p. 583.

work to include all the strains. No sharp difference in the ability of the strains to fix complement was manifested, as the serums were increased in dilution.

It would seem, therefore, that staphylococci are able to fix complement in more or less the same degree. Further, the reaction is a specific one for antigens prepared of streptococci and *B. friedländer* were unable to prevent hemolysis. But no evidence is given of a possible classification of staphylococci by complement fixation—either in a qualitative or quantitative way.

This is not in the least surprising, however, when we recall that complement fixation does not show divisions into groups with those cocci which have been proved to be of different serologic types by agglutination reactions.

AGGLUTINATIONS

The agglutination reactions of the staphylococci have been studied by several investigators. Kolle and Otto⁵⁰ found that immunized serum distinguished the pathogenic from the nonpathogenic forms. This was confirmed by Klopstock and Bockenheimer,^{51a} Van Durme,⁵¹ Proscher,⁵⁴ Kutscher and Konrich,⁵² Veiel,⁵³ Fraenkel and Baumann⁵⁶ and Montegazza.⁴⁹ Trincas⁵⁷ states that serum prepared with hemolytic strains shows strong agglutination with hemolytic strains, and slight agglutination with nonhemolytic strains; and vice-versa. Walker and Adkinson⁵⁸ found that an aureus immune serum would agglutinate aureus and not albus strains; and that an albus immune serum would agglutinate albus and not aureus strains.

Our object was to group staphylococci by agglutination into as many serologic groups as would evidence themselves, without regard to virulence or pigment. The same serums used in the complement-fixation test were used for agglutination, and the same antigens also, except that they were diluted until their turbidity equaled that of the Dreyer standard for the typhoid group agglutinations. The agglutinations were set up in serum dilutions of 1:10 and going as far as was necessary to include the agglutination titer of the respective serums. The serum dilutions and antigens were added in 0.5 c c amounts each, and incubation was effected in a water bath at 56 C. for 16 hours.

In table 12 the figures represent the dilution at which final agglutination was observed with naked eye. There was present in the serums a proagglutinoid zone.

An analysis of the table shows that serum A1 agglutinates strains A1, F1, P2, S2, T2, T5, T8, T9, X, C15, C16 and C18. Serums A5,

^{50a} Centr. f. Bakt., 1903, 34, p. 437.

⁵⁴ Arch. f. klin. Chir., 1903, 72, p. 325.

⁵⁵ München. med. Wehnschr., 1904, 51, p. 13.

⁵⁶ Ibid., 1905, 52, p. 937.

⁵⁷ Biochem. Centralbl., 1908, 8, p. 609.

⁵⁸ Jour. Med. Res., 1917, 35, p. 373.

P1, and L1 agglutinate strains A2, A3, A5, H2, P1, P3, P4, P5, T1, T2, T3, T6, T9, X, J1, and L1. Serum T9 agglutinates P3, T2, T9 and X. Serums A5, P1 and L1 are unquestionably the same since they give the same reactions. It will be noted that strains T2, T9 and X are agglutinated by all the serums, and P3 by all the serums except A1. Aside from these atypical agglutinations, the strains fall definitely with one serum. Apparently, then, the agglutination tests give the following grouping:

I.—A1, F1, P2, S2, T5, T8, C15, C16, C18.

II.—A2, A3, A5, H2, P1, P4, P5, T1, T3, T6, J1, L1.

III.—T2, T9 X and possibly P3.

TABLE 13
RESULT OF ABSORPTION TESTS ANTIGENS *

	Serum Absorbed With						
	A 1-A 1	A 1-X	T 9-P 3	T 9-T 2	L 1-A 5	L 1-P 3	L 1-T 9
A 1.....	0	4800	—	—	—	—	—
A 2.....	—	—	—	—	0	300	600
A 3.....	—	—	—	—	0	300	600
A 5.....	—	—	—	—	0	300	600
F 1.....	0	+	—	—	—	—	—
H 2.....	—	—	—	—	600	300	600
P 1.....	—	—	—	—	0	300	+
P 2.....	0	+	—	—	—	—	—
P 3.....	—	—	0	2400	600	0	1200
P 4.....	—	—	—	—	0	300	+
P 5.....	—	—	—	—	0	300	+
S 2.....	0	+	—	—	—	—	—
T 1.....	—	—	—	—	0	300	+
T 2.....	—	0	0	0	300	0	0
T 3.....	—	—	—	—	0	300	+
T 5.....	0	+	—	—	—	—	—
T 6.....	—	—	—	—	0	300	+
T 8.....	0	+	—	—	—	—	—
T 9.....	0	0	0	0	300	0	0
X.....	600	0	0	150	600	0	0
C 15.....	0	+	—	—	—	—	—
C 16.....	0	+	—	—	—	—	—
C 18.....	0	+	—	—	—	—	—
J 1.....	—	—	—	—	0	300	+
L 1.....	—	—	—	—	0	600	1200

* — indicates that strain did not agglutinate prior to absorption; figures represent dilution of final positive agglutination; + indicates no test. All controls were negative.

ABSORPTION TESTS

In order to further identify the groups suggested by the agglutination reactions absorption tests were conducted, employing the technic of Small and Dickson.⁵⁹ One c c of a 1:10 dilution of the immune serum was mixed with 4 c c of the concentrated antigen in a sterile centrifuge tube. This amount of the antigens was found sufficient to

absorb the homologous agglutinins after 4 hours' incubation at 37 C., the tubes being shaken at half-hour intervals. After this period of incubation the tubes were centrifugalized and the supernatant serum dilution (1:50) was drawn off and agglutinations carried out as described.

Serum A1 was absorbed with strain A1 and X; serum T9 with P3 and T2; serum L1 with A5, P3 and T9, and agglutinations performed against the antigens which agglutinated with the respective serum before absorption. The results are presented in table 13.

The absorption tests confirm the groups found by agglutination. Group 1 remains as was found, but in group 2, H2, is placed in a subgroup because although it agglutinates with the same serums as A5, absorption by A5 does not remove agglutinations for H2. In group 3, P3 is placed in a subgroup. P3 removes agglutinins for all members of group 3, but the other members of group 3 do not remove agglutinins for P3.

Revising our classification, then, we would have:

Group 1	Group 2	Group 3
A 1	A 2	T 2
F 1	A 3	T 9
P 2	A 5	X
S 2	P 1	Subgroup
T 5	P 4	P 3
T 8	P 5	
C 15	T 1	
C 16	T 3	
C 18	T 6	
	J 1	
	L 1	
	Subgroup	
	H 2	

DISCUSSION OF SEROLOGIC REACTIONS

The use of complement fixation in determining types among the staphylococci appears to be worthless. Although staphylococci do fix complement, no grouping appeared possible, either quantitatively or qualitatively. Nor is this surprising—on the contrary, it is more or less what was to be expected. Complement fixation has been disappointing in its inability to differentiate types—probably because the immunity established although specific for the particular species is general and not sufficiently specialized to detect individual types.

Agglutination, however, has already been proved to be an efficacious means of detecting types. Furthermore, agglutination is a fixed quality, and one which is considered reliable. So that, when the statement is

made that virulent types agglutinate only with serums prepared from virulent strains, there must be an error somewhere. The properties of virulence are obviously among the most unstable of bacterial characters. Culture on laboratory mediums renders a virulent strain nonpathogenic in a very short time. Yet it is scarcely conceivable that the immunity reactions are as readily modified. By way of illustration: Strain L1, which was distinctly pathogenic, was used for the preparation of immune serum before it could have undergone avirulence; but its serum did agglutinate other strains, including A5, P3 and T9, all 3 of which were proved nonpathogenic. It may be that A5, P3 and T9 were pathogenic at some time or another, but at the time the test was made they were not pathogenic. It seems clear to us that virulence does not dictate the group into which a staphylococcus shall fall.

Nor does it seem plausible that hemolytic activity is the basis of agglutination grouping. We have been unable to obtain absolutely non-hemolytic cultures, and have been unable to establish this point conclusively. However, we were able to get these groups among hemolytic organisms, whereas if hemolysis were the fundamental of the grouping, we should have obtained agglutination of all our strains by all our serums.

Regarding the association of pigment and agglutination, this much can be said: Occasionally, there may develop on a plate streaked with a pure culture, colonies varying appreciably in intensity of pigment, from which, as Sullivan⁶⁰ has shown, quite distinct types may be derived by selection of the extremes. Yet it does not seem probable that the parent strain in such a case would vary from its successor in its agglutination reactions. More relevant, however, strain J1, which is an albus, did agglutinate with serums A5, P1 and L1, which were prepared from antigens of varying shades of orange. An analysis of the pigment and agglutination tables, with this one exception cited, bears out the contention of Walker and Adkinson⁵⁸ in a general way. The members of group 1 are of a light pigment—either white or of a light shade of yellow, which without the refined technic of Winslow and Winslow²⁸ would easily be called a white.

A study of the tables of the different biochemical reactions shows no definite relationship between the agglutination groups and these reactions. In a very general way Group 1 seems to contain the less active strains, but it also contains some rather active strains. Groups 2 and 3 possess none of the light pigmented nor any of the less active strains.

⁶⁰ Jour. Med. Res., 1905, 14, p. 109.

These immunologic groups will perhaps explain the variations experienced in curative and prophylactic inoculations of either the organisms or serum. Stock vaccines, for example, will not necessarily be specific, nor will immune serum prove to be efficacious unless it falls into the same group. But having determined the group or type of staphylococcus under question, we can employ specific material either prophylactically or curatively.

CONCLUSIONS

Staphylococci produce a hemolytic substance in broth which appears on the 6th day, reaches a maximum at the 9th or 10th day and then disappears between the 13th and 16th day.

This hemolytic substance is thermolabile, is unaffected by the presence of carbohydrates and appears to be associated with proteolysis and possibly autolysis.

All cultures of staphylococci isolated during the course of this investigation appear to be hemolytic—only the time of its manifestation is in some cases considerably delayed.

Hemolytic cultures did not lose their hemolytic powers by continued transplantations into blood-free mediums for a period of more than four months.

Hemolytic activity shows no relationship to any of the biochemical reactions studied.

Staphylococci fix complement specifically, but cannot be classified by such an expedient.

Three groups seen definable of the 25 strains studied by agglutinations and absorption test, with 2 ill-defined subgroups—one each under group 4 and group 3.

These groups apparently bear no relationship to virulent hemolysis or biochemical activity. Group 1 appears to include the light pigmented and less active strains.

These groups may account for the variations experienced in the past in the use of serum and vaccines.

THE HECHT-WEINBERG-GRADWOHL TEST

STUDIES ON THE SERODIAGNOSIS OF SYPHILIS I.

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The discovery of the "complement-fixation reaction" (Bordet-Gengou phenomenon) by Bordet,¹ opened a new epoch in the serodiagnosis of the infectious diseases, and offered as well a means by which specific antibodies could be demonstrated. Wassermann and his co-workers,² shortly after the announcement of Bordet's discovery, applied the principle of complement fixation to the serodiagnosis of syphilis. They assumed that patients suffering from this infection carry specific antibodies in their blood, which, when brought in contact with the specific antigen, form a complex that absorbs or fixes complement. Their efforts were rewarded by most encouraging results, and stimulated other workers to make investigations along similar lines. It was soon demonstrated that the so-called syphilitic antigen need not contain spirochetal substance, but that certain tissue extracts (lipoidal in character) filled the antigenic requirements fully as well as the Wassermann antigen prepared from tissue rich in spirochetes. This proved that the reaction does not depend on a specific antigen in the true sense of the term, and the interesting question arose whether the bodies in the serum of syphilitic persons were true antibodies against *spirochaeta pallida* or other substances, possibly metabolites, produced in the body through the ravages of the disease. Probably the latter view has more evidence in its favor, although the question is far from being settled. Whatever may be the nature of the substances in the serum of syphilitic persons which enter the reaction, we know that they are not particularly thermostable, and in the process of "inactivating" such serums by heating even at 56 C. quite a decided destruction of the "fixing bodies" occurs (Noguchi³). To overcome this particular objection to the original Wassermann technic modifications of the method have been suggested. Among these that of Hecht and Weinberg⁴ has perhaps received the most attention, especially a modifi-

Received for publication, May 24, 1922.

¹ Ann. de l'Inst. Pasteur, 1900, 14, p. 257; 1901, 15, p. 290.

² Deutsch. med. Wchnschr., 1906, 32, p. 745.

³ Serum Diagnosis of Syphilis, 1912, p. 96.

⁴ Wien. klin. Wchnschr., 1909, 22, p. 256.

cation suggested by Gradwohl.⁵ Gradwohl, by his modification, claims that a positive reaction may be obtained with the serum of syphilitic persons in a larger number of cases (15% or higher) than by the usual Wassermann technic, which he employed. His claims are supported by certain other workers (Blaivas,⁶ Christian⁷).

Most serologists have occasionally observed that the serum from certain syphilitic persons fails to give positive reactions by the former test method. Any practical modification which will give positive reactions in such cases is quite worthy of investigation and adoption if its superior merits are proved. On the other hand, the tendency of certain serologists to employ oversensitive tests which may give pseudo-positive reactions is a dangerous procedure, and one which should be discouraged. It is quite difficult to delimit the borderline between the nonsyphilitic and the syphilitic person. Supersensitive laboratory tests on nonsyphilitic serums in certain cases may give results which pass over to the syphilitic side. This is especially true when the worker is in pursuit of those who clearly show syphilitic conditions, but whose serums fail to give a positive reaction with the usual methods. The nonreacting syphilitic may not even carry "syphilitic fixing bodies" in his serum. It is well known that there is a decided quantitative difference existing between the different positive serums. If these "fixing bodies" are metabolites, they may arise anew, or may be normal substance markedly increased in amount during the course of the disease. In the latter case, it is quite conceivable that certain nonsyphilitic persons may naturally have an abnormally large content of these substances in their blood, while, on the contrary, known syphilitic persons may fail to elaborate these bodies and show only a very low content, even below the average norm for the healthy person. Therefore members of either group may be found on opposite sides of an arbitrarily established borderline, where clinically they do not belong. Such possibilities must be considered, and caution must be exercised by the serologist not to place the stigma of syphilis on the noninfected by applying a test which picks up the occasional elusive syphilitic serum, but also gives a positive reaction in certain nonsyphilitic persons.

As to the criteria which should determine the syphilitic from the nonsyphilitic persons, no common agreement exists. By cooperative studies among clinicians, pathologists and serologists, progress may

⁵ Jour. Am. Med. Assn., 1914, 53, p. 240; 1917, 68, p. 514. Am. Jour. Syph., 1917, 1, p. 450.

⁶ Jour. Lab. and Clin. Med., 1919, 5, p. 244.

⁷ Am. Jour. Syph., 1919, 3, p. 613.

be made toward that ultimate end. In the absence of a standard method for the serum diagnosis of syphilis, a multitude of modifications of the original Wassermann technic have come into existence. Many of these modifications are erroneous in conception, even conflicting with the established laws of serology, consequently leading to false results. Owing to the fact that no absolute criterion exists by which the presence or absence of syphilis may surely be determined in a person, the acceptance of any particular laboratory test as an exact basis to evaluate another method must be dependent on a most critical analysis. The basic method applied in such studies may be faulty, and give rise to misleading conclusions. Recognizing these conditions, the investigator should use as a basis a method which experience has shown gives trustworthy results as a diagnostic means.

In undertaking the present study, the authors have attempted to determine the comparative serodiagnostic relationship existing between a modified Wassermann technic,⁸ which has been found quite satisfactory, and the Hecht-Weinberg-Gradwohl technic, when applied to the serum of a group of general hospital cases. In carrying out the latter technic, utmost care was exercised to conform with the Gradwohl test as published. The radical difference between the two tests lies in the source of the complement and the antishoop immune body. In the modified Wassermann technic employed, the serums were inactivated by heating in the water bath for 30 minutes at 54 to 56 C. to destroy the natural complement present. An accurately standardized amount of complement was then added in the test, and finally, a constant amount of sensitized sheep corpuscles, as an indicator for the degree of complement fixation. In the Hecht-Weinberg-Gradwohl modification, fresh active serum of the patient is used, advantage being taken of the presence of complement and natural antishoop hemolytic immune body (amboceptor) which most persons possess. When both complement and hemolytic immune body are present the compensating phenomenon of von Dungern⁹ and of Morgenroth and Sachs¹⁰ is applied to determine the amount of serum which will just lake a given amount of the red blood cells of the sheep. This is called the "hemolytic index," and is expressed numerically, depending on the amount of 5% suspension of sheep blood corpuscles completely laked by 0.10 c.c. of the fresh human serum under specified conditions. If

⁸ Wood, Vogel and Famulener: *Laboratory Technique*, 1922, p. 247.

⁹ München. med. Wehnschr., 1900, 47, p. 677.

¹⁰ Berl. klin. Wehnschr., 1902, 39, p. 817.

the given amount of test serum is found to take 0.10 c c, or its multiple of sheep corpuscle suspension, the index is indicated by the corresponding number, i. e., 0.10 c c gives a "hemolytic index" of 1 . . . 0.4 c c of 4; etc. In case the hemolytic index is low, that is, under 4, the test is omitted as without value over the Wassermann method, and those serums which show no hemolysis are not applicable to the test. In each method the same antigen (acetone-insoluble antigen of Noguchi) was used, and in addition with the Wassermann method a cholesterinized antigen was used.

Two series of tests were carried out on serums submitted to the laboratory for routine tests. All were used in the fresh condition, some only shortly after the blood was taken, none after 24 hours. These were kept in the icebox when not tested immediately after drawing blood.

In the first series of 50 serums, only 7 were found to possess sufficient hemolytic power for the advantageous application of the test. Of these, only one gave positive results with both methods; one gave a questionable positive with the Hecht-Weinberg-Gradwohl method and negative with the Wassermann method with the acetone-insoluble antigen, but a low positive with the cholesterinized antigen. The history of this case might be considered suspicious of syphilis, although no definite clinical evidence was shown. The remaining 5 serums gave negative results with both methods. Since the sheep corpuscles used in this test were from an old laboratory animal, which had been used for bleeding purposes over a considerable time, it was thought that perhaps its cells had become resistant to hemolysis by the human system employed. This possibility might account for the relatively small percentage of human serum found suitable for test in the foregoing series.

In a similar manner a second series of tests on 100 serums was undertaken, using the blood from a sheep which previously had been bled little. Of these, only 34 were found suitable (hemolytic index 4 or higher) for testing. Twenty-nine of this group gave negative reactions, both with the Hecht-Weinberg-Gradwohl and the Wassermann method when the acetone-insoluble antigen was used, but one of these gave a positive (+ + + +) reaction by the latter method with the cholesterinized antigen. Of the 5 which gave positive reactions with the Hecht-Weinberg-Gradwohl test, one serum (hemolytic index, 5) which was weakly positive, was negative in the Wassermann test with the acetone-insoluble antigen, but gave a doubtful reaction (\pm) with the

cholesterinized antigen—this was a treated case of syphilis. The serum which was negative with the acetone-insoluble antigen by both methods, but strongly positive (+ + + +) with the cholesterinized antigen by the Wassermann method, had a hemolytic index of 4. This patient gave a history of having contracted syphilis 10 years before and had undergone hospital treatment, including treatment with arsphenamin. In this series, 6 patients had a syphilitic history. If a serum were negative by the Wassermann technic with the cholesterinized antigen, in all probability the patient was nonsyphilitic or at most "cured." Since this test with this antigen was considered as highly sensitive, it was used as a means of excluding nonsyphilitic persons.

Gradwohl states that 98% of patients have sufficient natural anti-sheep amboceptor and complement to carry out the test, while Christian⁷ found only 93.3% in his series. Wade,¹¹ in his studies on natural hemolysins in fresh human serum, found that the anti-sheep was absent in 16%, and that it showed great variability in concentration. In our small series we found that considerable discrepancies existed in the results when tested with the red blood cells of 2 different sheep. As the same serums were not tested in parallel with corpuscles from the 2 animals, it is not possible to say whether this was a coincidence or due to greater resistance of the erythrocytes of one of the animals. We suspect the latter. If a hemolytic index of 1 be accepted as a basis for test purposes, our results would show that 22% of the first and 10% of the second series would be unsuitable. But since Gradwohl states that his modification has no advantage over the Wassermann technic except in serums with a hemolytic index of 4 or over, we find that only 14% in the first and 34% in the second series fall into that category. As is evident, our results indicate that only a relatively small percentage of serums was applicable where we might expect the greatest value of the test. Also, since the relative amounts of anti-sheep immune body and complement in the serum of different persons are exceedingly variable, the accuracy of the complement fixation test may be questionable in some instances. This source of error has long been recognized by many serologists, and has been pointed out in particular by Ottenberg.¹²

SUMMARY

The number of serums (150) examined in the 2 series of tests herewith reported is quite inadequate for purposes of definite conclusions.

¹¹ Jour. Med. Research, 1916, 29, p. 113.

¹² Jour. Immunol., 1917, 2, p. 39.

However, we found that the reactions with the Hecht-Weinberg-Gradwohl test were hardly so marked as with the Wassermann technic, but the Gradwohl modification might be considered somewhat superior when relying only on the results with the acetone-insoluble antigen in each method. On the other hand, the use of the cholesterinized antigen with the Wassermann method showed a stronger reaction with each positive serum (with one exception), and further gave a strongly positive reaction in one case—tertiary syphilis—which was negative with the Hecht-Weinberg-Gradwohl technic. In this report all serums giving positive reactions came from patients who gave a clear clinical history of syphilis, with the exception of one who had a suspicious history.

THE MATHERS COCCUS IN THE THROAT IN INFLUENZA

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During February and March, 1922, an epidemic of so-called influenza occurred in Iowa City. The disease was highly contagious, of short duration, and was followed by few complications. A study was made of the bacterial content of the throats of the students of the second year class of the medical school, before, during, and for a considerable time after the epidemic. Cultures were also made from the tonsils and nasopharynx of patients sent to the hospital with influenza and of other students with similar symptoms.

In most cases the symptoms were marked from 3 to 5 days; many of the students remained in bed from 1 to 3 days; others continued to attend classes, although they had some fever and felt rather ill. In a few instances symptoms persisted for 3 weeks. There were three types of symptoms: nasal, laryngeal, and bronchial, all of which were associated with considerable lassitude and general malaise. The general symptoms were mild, however, in the laryngeal form, but the cough was persistent in most cases.

The epidemic began Feb. 18, when 2 students complained of a severe cold, and 42 students of a class of 74 had a similar attack before March 6. There was only an occasional case after this time, until April 5 to 8, when 7 students had a rather severe attack of coryza. Five of these did not have influenza during the first epidemic. Since this time there have been occasional cases of coryza among the students, but the symptoms have been different and the throat cultures have contained a variety of bacteria.

Blood counts were made on 5 students and 6 patients in the hospital with influenza; two students had white counts of 6,200 and 6,800, two hospital patients had similar counts, and one patient had a white count of 12,600. The counts of the others were within the normal range.

In making throat cultures, swabs were passed over the tonsils and posterior pharyngeal wall. The swabs were then washed in tubes containing 1 c c of salt solution. One to three loopfuls of this suspension

were added to melted tubes of agar, which were then poured into plates containing 1 c c of defibrinated sheep blood. After 24 and 48 hours' incubation, an estimate of the number and types of colonies was made. Records were kept of the 4 principal types: (1) Str. hemolyticus; (2) gram-negative cocci; (3) Str. viridans and pneumococcus, and (4) a peculiar green-producing streptococcus. Three colonies of the green-producing streptococci from each plate were streaked to blood agar slants, and tested for bile solubility and the fermentation of inulin. Agglutination tests were then made on the bile soluble strains with 3 types of antipneumococcus serum. Cultures were also made on Loeffler's medium from each student on 2 different occasions and examined for diphtheria bacilli. In the early part of the work cultures were also made on chocolate blood agar to determine the presence of *B. influenzae*.

TABLE 1
HEMOLYTIC STREPTOCOCCI FOUND IN THROATS OF STUDENTS

Time of Cultures	Total Cultures	Students Cultured	Cultures Containing Str. Hemolyticus
Before attack.....	23	17	8 (34%)
During attack.....	68	45	10 (15%)
After attack.....	104	55	33 (31%)
Those not affected.....	35	14	8 (22%)

Hemolytic streptococci were found one or more times in the throat of 33 students, and in 10 they were present at every examination. The number of hemolytic streptococci in the throat was often increased after the disease but in some instances the number was less during the acute stage. In 3 students they were found in small numbers before the attack, were absent during the attack, and were the predominant organism following the disease. From table 1 it appears that the number of these organisms in the throat was less during the acute stage of the disease than at other times. The number of cultures taken from the class of students with the number of times hemolytic streptococci were found are given in table 1.

Diphtheria bacilli were not found in two cultures taken from each student.

The influenza bacillus was isolated 8 times in 23 cultures from normal throats and 3 times in 19 cultures during the acute stage of the disease. The cultures taken during the acute stage were overgrown

with a green-producing streptococcus. The attempt to isolate the influenza bacillus was discontinued in the latter part of the work.

Staphylococci and gram-negative cocci were found in larger numbers in normal throats. In many cases none of these organisms was found during the acute stage in dilutions when there were isolated colonies on the plates. In a few cases gram-negative cocci were the predominant organisms after the attack.

Although no particular attempt was made to isolate the pneumococcus from each case, in those cases in which there were different types of green colonies, at least one of each kind was transferred to blood-agar slants. All strains which were soluble in bile were then typed with the antipneumococcus serums. Pneumococci were isolated 19 times from 15 students. Type 1 was found 3 times; twice from the same student at different examinations. Type 2 was found once and type 4, 15 times. Type 4 was isolated from 3 cases at 2 different examinations. The pneumococci were isolated more often from cases of slight pharyngitis occurring after the epidemic.

Direct smears and cultures on blood agar gave a peculiar green-producing streptococcus as the predominating organism during the first few days of the disease; it was isolated also from the conjunctival secretions in 2 cases with marked conjunctivitis. This streptococcus appears to be the same as that described by Mathers,¹ and more recently by others,² in connection with their work on influenza. A review and discussion of the literature concerning this streptococcus has been made by Tunncliff.³ This streptococcus has been found by Pilot and Pearlman⁴ in 16% of extirpated adenoids.

On the surface of blood agar the organism produces large, flat, moist, greenish colonies, with a tendency to coalesce. In the depth of the medium the colonies are larger than the common *Str. viridans*. The central colony has an indefinite outline, but this is surrounded by a definite brownish-green zone, and surrounding this there is a narrow hemolytic zone. The hemolytic zone usually becomes definite in 48 hours. The morphology of this organism is much like that of the pneumococcus. In strains from the sputum, milk, and inulin serum

¹ Tunncliff: Jour. Am. Med. Assn., 1918, 71, p. 1733.

² MacDonald: Brit. Med. Jour., 1919, 2, p. 481. Rosenow: Jour. Am. Med. Assn., 1919, 72, p. 31 and 1604. Jordan: Jour. Infect. Dis., 1919, 25, p. 28. Abstracts of foreign literature compiled by the British Research Committee in the Jour. Am. Med. Assn., 1918, 71, p. 1573.

³ Jour. Infect Dis., 1920, 26, p. 405.

⁴ Ibid., 1921, 29, p. 51.

water, a capsule could usually be seen. It was often difficult to find them in inulin serum water in which acid and coagulation had developed. Quite often a short chain was found in which only one or two cocci were stained. It was practically impossible to find them in coagulated inulin serum water after 5 days' incubation. The relation of this streptococcus to influenza is given in table 2. Only those plates on which there were between 50 and 500 colonies are included in this table.

The cultures taken 24 hours before were taken as controls and 3 of the persons with practically pure cultures at this time remained in bed the next day. All of the others complained of definite symptoms the following day, although they were more mild in those with few of these organisms in the throat. Among the 6 who did not have these cocci during the acute stage, there were 2 in which the hemolytic strepto-

TABLE 2
RELATION OF STREPTOCOCCUS TO INFLUENZA

	24 Hours Before Illness	1st and 2d Days of Illness	5-7 Days of Illness	4 Weeks or More After Illness	Those Not Affected or 5 Days Before Illness
In practically pure culture....	5	22	4	0	5
Predominant organism.....	1	14	5	2	2
Few.....	2	3	2	16	6
None.....	2	6	4	58	12

cocci were so numerous that other types could not be isolated. In another case a gram-negative coccus was the predominant organism. The majority of the colonies which developed in the remaining 3 were green-producing streptococci whose morphology and cultural characters were different. There were also many colonies of gram-negative cocci on 2 of these.

The fermentation reactions were tested of 2 typical colonies isolated from each of 34 persons, most of them from acute cases among the students, although representative strains from different stages of the disease—from those not affected and from influenza patients in the hospital—were included. The ability to ferment carbohydrates was tested in broth containing 1% of the sugar and 1% of Andrade indicator. Readings were made after 2 and 5 days' incubation. Dextrose, levulose, maltose, sucrose, and lactose were uniformly fermented; 3 strains, however, failed to ferment lactose. The majority of the cocci produced acid in inulin, salicin, and raffinose. Of 209 strains tested in inulin serum water, 173 produced acid. Mannite and dextrin were not

fermented. Milinska,⁵ working with apparently the same organism, found a few strains that did not ferment lactose, and some strains which fermented mannite. By their fermentation reactions he divides these cocci into 4 types.

The virulence of 8 strains for rabbits and mice was tested by intraperitoneal injections of 0.5 to 1 c c of 24-hour broth cultures into rabbits, and 0.1 to 0.3 c c into mice. All of the animals survived these injections. A rabbit injected with the growth from 2 blood agar slants died in 40 hours. Two rabbits injected with 0.5 c c of sputum from severe cases died in 48 hours. This streptococcus was isolated from the heart blood in both cases, but in one a type 4 pneumococcus was the predominant organism. Three of 4 mice injected with small amounts of sputum died in 24 hours. One of these was injected with the same sputum that was injected into the rabbit from which type 4 pneumococcus was isolated. The pneumococcus was the only organism isolated from the mouse. The streptococci were found in pure culture in the other mice. From these experiments it appears that the cocci are not highly virulent for mice and rabbits.

CONCLUSIONS

Hemolytic streptococci were present in 47% of the students examined 2 or more times, and appeared in smaller numbers during an attack of influenza. The pneumococcus was isolated from 15 students, and type 4 was found in 12 of these. The influenza bacillus could be isolated more often from the normal throat than from the throats of influenza patients. A green-producing streptococcus resembling the pneumococcus in morphology but insoluble in bile was found to be the predominating organism in an influenza epidemic affecting 75% of a class of students. The same organism was found as the predominant one in the throats of other students with similar symptoms and in the throats of patients in the hospital with influenza. This coccus grows characteristically on blood agar and uniformly ferments dextrose, levulose, maltose, sucrose, and lactose. In most cases inulin, salicin, and raffinose are also fermented. Dextrin and mannite are not fermented. It is only slightly virulent for mice and rabbits. The relation of this organism to the epidemic is very interesting. For convenience of discussion and because of the early study by Mathers of this organism in relation to influenza, the name of *Streptococcus mathersi* is suggested for it.

⁵ Przegląd Epidemiol., 1920, 1, p. 29; abst., Offic. Inter. d'Hygiene Publique, 1922, 14, p. 171.

THE INCUBATION PERIOD OF TYPHOID FEVER

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In most cases of typhoid fever the incubation period cannot be determined accurately, as the precise date of infection is unknown. There are, however, cases in the literature in which the time of infection is definitely known and in which, therefore, the distribution of the incubation period can be determined. Certain of these are treated in the present paper.

In an epidemic, described by Sawyer,¹ caused by the eating of infected spaghetti at a church dinner the distribution of the incubation periods of definite and of doubtful cases was as given in table 1:

The definite and doubtful cases do not therefore differ significantly in length of incubation period.

In an epidemic reported by Cumming,² due to the contamination of ice cream by a typhoid carrier, the distribution of incubation periods is given in table 2.

The mean of the fatal cases is 5.67 days, and the standard deviation is 0.471, while the mean of the nonfatal cases is 7.2 and the standard deviation is 1.887. By Pearson's goodness of fit test the probability that the fatal and nonfatal cases are random samples of the same population is 0.8. The difference in the means cannot, therefore, be considered significant on the basis of the present small numbers. The mean of the combined cases is 7 ± 0.26 days, and their standard deviation 1.84 ± 0.18 days.

Other cases due to infection of food have been reported by Schenck.³ He reports nineteen cases with an incubation period of 12-16 days. Waterman⁴ reports seventeen cases, 13-22 days. Pixley⁵ reports two cases with an incubation period of 2 weeks; also 3 cases due to infected water with an incubation period of three weeks.

Received for publication June 8, 1922.

¹ Jour. Am. Med. Assn., 1914, 73, p. 1537.

² Jour. Am. Med. Assn., 1917, 68, p. 1163.

³ Jour. Am. Med. Assn., 1917, 69, p. 1402.

⁴ Public Health, 1917, 5, p. 157.

⁵ N. Y. Med. Jour., 1913, 48, p. 328.

Laboratory infections are reported by Sawyer⁶ (incubation period of 9 days), Hall⁷ (21 days) and Boldman and Noble⁸ (2 weeks).

In a water-borne epidemic at Mankato, Minn., reported by Hill,⁹ there were 21 cases in which the patient had been in the city only one day and in which the incubation period was therefore definitely known.

We may similarly test whether male and female distributions differ significantly, the probabilities in this case that the distributions are

TABLE 1
NUMBER OF CASES OF TYPHOID CAUSED BY INFECTED SPAGHETTI SERVED AT A
CHURCH DINNER

Days	Definite	Doubtful	Total
3	1	.	1
4	3	1	4
5	12	.	12
6	19	.	19
7	13	.	13
8	7	3	10
9	2	3	5
10	2	.	2
11	5	.	5
12	2	.	2
13	.	1	1
14	2	.	2
15	3	.	3
16	2	.	2
17	1	.	1
18	1	.	1
19	2	1	3
20	.	.	.
21	2	.	2
22	.	.	.
23	1	.	1
24	1	.	1
25	1	.	1
26	1	.	1
27	.	.	.
28	.	.	.
29	1	.	1
	84	9	93
		Mean	Standard Deviation
Definite		9.52 \pm .43	5.82 \pm .30
Doubtful		9.67	3.94
Total		9.54 \pm .39	5.63 \pm .28

random samples of the same population being 0.57: We cannot, therefore, conclude from this material that there is a significant difference in incubation period between males and females, although the fact that 10 patients who had been in the city for from 2 to 4 days showed the same tendency for females to have a longer incubation period strengthens the probability of its significance. The question deserves further investigation.

Jour. Am. Med. Assn., 1915, 64, p. 2053.

Buffalo Med. Jour., 1918-1919, 74, p. 12.

⁸ N. Y. Med. Jour., 1911, 94, p. 1313.

⁹ Jour. Infect. Dis., 1911, 9, p. 410.

TABLE 2
INCUBATION PERIODS IN EPIDEMIC OF TYPHOID CAUSED BY INFECTED ICE CREAM

Days	Number of Cases		Total
	Fatal	Nonfatal	
5	1	3	4
6	2	5	7
7	.	7	7
8	.	1	1
9	.	1	1
10	.	1	1
11	.	1	1
12	.	1	1
	3	20	23

TABLE 3
WATER-BORNE EPIDEMIC OF TYPHOID AT MANKATO, MINN.

Days	Number of Cases		Total
	Male	Female	
5	1	1	2
6	.	.	.
7	1	.	1
8	2	.	2
9	.	.	.
10	1	1	2
11	.	1	1
12	1	.	1
13	.	.	.
14	.	1	1
15	1	1	2
16	.	1	1
17	3	.	3
18	1	2	3
19	.	.	.
20	1	.	1
21	.	.	.
28	.	.	.
29	.	1	1
	12	9	21
Mean	Male 12.83	Female 15.11	Total 13.81 \pm .83
Standard deviation.....	4.88	6.30	5.65 \pm .59

TABLE 4
DISTRIBUTION OF INCUBATION PERIODS OF TYPHOID EPIDEMIC CAUSED BY WATER ON AN EXCURSION STEAMER

Days	Number of Cases	Days	Number of Cases
7	1	19	.
8	.	20	2
11	.	21	.
12	1	25	.
13	.	26	1
14	2	27	.
15	2	36	.
16	1	37	1
17	.	38	1
18	1	..	.
	8		5
13			

In an epidemic reported by Lumsden,¹⁰ caused by contaminated water on an excursion steamer, the distribution of incubation periods was as given in table 4:

The mean incubation period was 19.38 ± 1.66 ; the standard deviation was 8.85 ± 1.17 .

In an epidemic reported by Ferguson,¹¹ caused by the contamination of the water supply at the Old Salem Chautauqua by a flood 4 days before the close of the Chautauqua, and in which, therefore, the incu-

TABLE 5
DISTRIBUTION OF INCUBATION PERIODS IN EPIDEMIC AT THE OLD SALEM CHAUTAUQUA

Days	Cases	Days	Cases
7-10	1	23-26	9
8-11	2	24-27	3
9-12	3	25-28	3
10-13	6	26-29	3
11-14	16	27-30	2
12-15	6	28-31	3
13-16	10	29-32	2
14-17	10	30-33	2
15-18	16	31-34	1
16-19	18	32-35	1
17-20	9	33-36	4
18-21	10	34-37	1
19-22	11	35-38	1
20-23	7	36-39	1
21-24	10	37-40	1
22-25	10	1
	145		36
181			

bation period is known within 4 days, the distribution including only certainly primary cases, is as given in table 5.

Assuming that the infections occurred at the middle of the 4-day period of exposure, the mean incubation period is 19.50 ± 0.31 days and the standard deviation 6.10 ± 0.21 . The moments about the mean are:

$$\pi_2 = 37.249; \pi_3 = 183.812; \pi_4 = 4708.17.$$

$$\beta_1 = .6538; \beta_2 = 3.393.$$

The frequency curve representing the distribution of incubation periods is therefore Pearson's Type I. Working out the constants we find for its equation:

$$y = 12.396 \left(1 + \frac{x}{5.955}\right)^{.8573} \left(1 - \frac{x}{40.040}\right)^{5.7641}$$

¹⁰ Pub. Health Repts., No. 104.

¹¹ Ill. Med. Jour. 1916, 30, p. 247.

The mode is at 15.55 ± 0.96 days; the skewness is $+0.65 = 0.14$. This high positive skewness is also apparent in the other epidemics so far as the smallness of the numbers involved permits judgment as to the form of the distribution. It is, therefore, probably a general characteristic of the incubation period in typhoid fever, and renders more probable the isolated long incubation periods which are sometimes recorded and which might otherwise be attributed to a later unrecognized exposure. The lower limit of the curve is 9.59 days, the upper limit 55.59 days.

The curve, shown in chart 1, is evidently a good representation of the original data. By Pearson's test of goodness of fit the probability

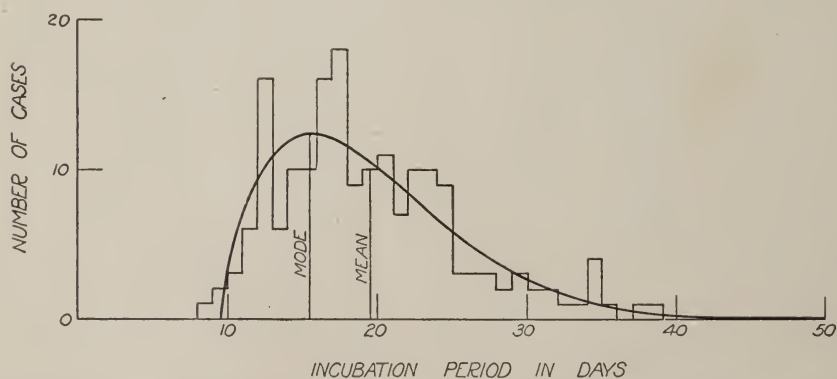


Chart 1.—Histogram and fitted curve for distribution of incubation period of typhoid.

that divergences due to random sampling shall give a worse fit $P_s = 0.49$, i. e., in one-half the cases a sample would diverge from the population more than the present data do from the fitted curve. As the standard deviation is not abnormally high, the uncertainty as to incubation period has probably not affected the distribution appreciably.

SUMMARY

The incubation period of typhoid fever is highly variable, ranging in the cases here recorded from 3 to 38 or 40 days. The mean incubation period in different epidemics ranges from 7 ± 0.26 to 19.50 ± 0.31 days, differing significantly from one epidemic to another. The

standard deviations range from 1.84 ± 0.18 to 8.85 ± 1.17 . The distributions are positively skew.

The results here obtained are consonant with the belief that the length of the incubation period depends in part on the virulence of the infection. The means of the epidemics due to infected water are 13.81 ± 0.83 , 19.38 ± 1.66 , and 19.50 ± 0.31 , whereas the means for those due to infected food, in which the dose was probably more massive, are 7 ± 0.26 and 9.54 ± 0.39 .

19.38

THE RELATIVE EFFECT OF CERTAIN TRIPHENYL-METHANE DYES UPON THE GROWTH OF BACILLI OF THE COLON GROUP IN LACTOSE BROTH AND LACTOSE BILE

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The use of lactose bile as a medium for the preliminary selective cultivation of organisms of the colon group was first suggested by Jackson in 1906;¹ most of the earlier workers who used this medium recommended a 10% solution of dried oxgall as its basis. This medium eliminated many organisms likely to interfere with the isolation of bacteria of the colon group, but it also inhibited the weaker members of the colon group itself. Melia² and Levine³ have shown that a 5% solution of bile is free from this objection and is, in general, stimulating rather than inhibitory for colon group organisms.

Following the remarkable researches of Churchman,⁴ Hall and Elefson⁵ suggested the use of gentian violet as an aid in the selective cultivation of *Bacterium coli* and its allies; while Bronfenbrenner, Schlesinger and Soletsky⁶ and Muer and Harris⁷ have, respectively, recommended rosolic acid and brilliant green for the same purpose.

The present study was concerned with a determination of the relative influence of these 3 dyes on the development of colon group organisms in lactose bile and lactose broth, respectively. The 5 following organisms were used for the test: *Bact. acidi-lactici*, *Bact. aerogenes*, *Bact. coli* (2 strains), *Bact. pneumoniae*. They were all obtained from the collection now maintained by the Society of American Bacteriologists in Washington. Transfers were made from 24-hour agar slants with a straight wire, and incubation was at 37 C. for 96 hours.

Received for publication, June 23, 1922.

¹ Biologic Studies by Pupils of William Thompson Sedgwick, 1906.

² Am. Jour. Public Health, 1915, 5, p. 1168.

³ Abstracts Bacteriol., 1922, 6, p. 37.

⁴ Jour. Exper. Med., 1912, 16, p. 221 and p. 822; 1913, 17, p. 373.

⁵ Jour. Bacteriol., 1918, 3, p. 329.

⁶ Ibid., 1919, 5, p. 79.

⁷ Am. Jour. Public Health, 1920, 10, p. 874.

The lactose bile was prepared according to the method described by Muer and Harris ⁷ as in use at the Mount Prospect Laboratory, except that the dye was not added until after the medium was complete in other respects, because of the varying dilutions which were used in the tests; filtering was done with filter paper. Sodium cholate (Merck) was also used in place of dried oxgall.

The lactose broth was prepared by adding 8 gm. of dehydrated nutrient broth (Difco) and 10 gm. of lactose to each liter of distilled water, warming to aid solution and filtering through filter paper.

The dyes were kept in a 1% stock solution, the brilliant green in water, the gentian violet and rosolic acid in alcohol, because of their slower solubility in water. For use in the higher dilutions these stock solutions were further diluted with distilled water to 1 part in 10,000. The gentian violet and brilliant green were from Eimer and Amend and the rosolic acid from Merck and Company.

TABLE 1
AVERAGE AMOUNT OF GAS FORMED IN FORTY-EIGHT HOURS BY FIVE STRAINS OF COLON GROUP ORGANISMS

	P _H			
	5	6	7	8
Broth, 48 hours.....	12	15	20	25
Broth, 96 hours.....	17	18	25	25
Bile, 48 hours.....	..	28	43	46
Bile, 96 hours.....	..	33	49	60

We first desired to study the influence of the bile and broth mediums without the dyes but adjusted to various acidities, and the results of these preliminary tests are indicated in table 1. Growth occurred in practically all cases so that the amount of gas formed is used as our criterion of viability. The results presented are the averages for the 5 strains studied.

It appears, in the first place, that the bile medium (or more accurately the sodium cholate medium) was distinctly more favorable than the lactose broth, since at every P_H value the bile medium showed more gas in 48 hours than the broth medium did in 96 hours. In the second place, the amount of gas produced was progressively increased by an increasing alkalinity of the medium up to P_H 8.

We next proceeded to a study of the inhibitive effect of the 3 dyes in mediums adjusted to a P_H of 7. The inhibitive concentrations, as determined by a wide range of tests extending to dilutions above and below the critical point, are indicated in table 2.

SUMMARY

In lactose broth rosolic acid is inhibitive in a concentration of 1 part in 1,000 for all 5 organisms studied; and in the lactose bile (or sodium cholate) medium the action of this dye is exactly the same.

Gentian violet is from 5 to 50 times as toxic as rosolic acid in the broth medium, the individual organisms showing marked variations in susceptibility. In the bile salt medium, on the other hand, the toxicity of gentian violet is exactly the same as that of rosolic acid, 1 part in 1,000 being inhibitive.

With brilliant green the most startling differences appear. This dye inhibits the organisms studied in concentrations between 1:100,000 and 1:1,000,000 in the broth medium (the strains of *Bact. aerogenes*

TABLE 2
RECIPROCAL OF INHIBITIVE CONCENTRATIONS OF CERTAIN DYES

Organism	Lactose Broth			Lactose Bile		
	Rosolic Acid	Gentian Violet	Brilliant Green	Rosolic Acid	Gentian Violet	Brilliant Green
<i>Bact. coli</i> a.....	1000	50,000	1,000,000	1000	1000	1000
<i>Bact. coli</i> b.....	1000	10,000	500,000	1000	1000	1000
<i>Bact. acid-lactici</i>	1000	10,000	1,000,000	1000	1000	1000
<i>Bact. aerogenes</i>	1000	5,000	100,000	1000	1000	500
<i>Bact. pneumoniae</i>	1000	10,000	100,000	1000	1000	500

type being more resistant than those of *Bact. coli* type). In the presence of the bile salt, however, the extreme toxicity of brilliant green wholly disappears, *Bact. aerogenes* and *Bact. pneumoniae* growing even in a concentration of 1 part in 500.

Opportunity has been lacking to follow out the chemical problems involved in these phenomena; the facts are therefore presented without further comment in view of their substantial importance in relation to the choice of mediums for selective cultivation based on the use of the triphenylmethane dyes.

EFFECT OF INJECTION OF ACTIVE DEPOSIT OF RADIUM EMANATION ON RABBITS

WITH SPECIAL REFERENCE TO THE LEUKOCYTES AND
ANTIBODY FORMATION

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Previous studies¹ on the effects of benzene, roentgen ray and thorium X on antibody formation led to an interest in the effect of radium in this respect. Radium salts in soluble form when injected in practicable doses were found to be eliminated too rapidly to produce any effect. On account of the ease of injection of the "active deposit" of radium emanation as used by Baggs² this form of radium seemed feasible for our work.

Baggs³ studied the changes produced by the intravenous and subcutaneous injection in the white rat of solutions of an active deposit of radium emanation and Theis and Baggs⁴ the effect of the intravenous injection in dogs. Doses from 2.6 to 10.6 millicuries given intravenously to rats did not cause death of the rats within a month. An increase from 10.6 to 11.2 millicuries was fatal in 2.5 days and all doses above this caused immediate acute effects. Subcutaneously, doses up to 10 millicuries were not fatal, while 17 millicuries killed in 5 days. After injection of active deposit of radium emanation the radioactive substance diffuses throughout the body and causes changes in the liver, lungs, kidneys, suprarenals, spleen, marrow, brain and vascular system. The liver, even after small doses subcutaneously, presents a fatty change with many giant cells and hyperchromatic nuclei which persist for a comparatively long time. After large doses, congestion and hemorrhages are frequent in practically all organs, and the animals may die with symptoms of enteritis. The most frequent change in the kidney is a granular degeneration and erosion of the tubular cells. Destruction of the cells of the marrow occurs, and in the spleen there may be found congestion with hemorrhages and destruction of red corpuscles. Intravenous injections affect the lungs more severely than the subcutaneous, producing proliferation and desquamation of the epithelial cells of the bronchi, marked edema, congestion and hemorrhage. The reaction in the tissues from radium injections is like that from radium applied externally.

Dogs tolerated as high as 146.4 millicuries in a single injection or a total of 338.4 millicuries in four intravenous injections. Large injections produce a

Received for publication, April 20, 1922.

¹ Hektoen: *Jour. Infect. Dis.*, 1916, 19, p. 69; 1915, 17, p. 415; 1918, 22, p. 28; 1920, 27, p. 23. Corper: *ibid.*, 1920, 26, p. 330.

² *Amer. Jour. Anat.*, 1922, 30, p. 133.

³ *Jour. Cancer Research*, 1920, 5, pp. 1 and 301.

⁴ *Jour. Biol. Chem.*, 1920, 41, p. 525.

considerable reduction in the number of leukocytes, sometimes as much as 80% of the total number, but the simultaneous reduction in red corpuscles is less, about 25%. The principal organs showed considerable congestion; the liver a general fatty and granular change, the kidneys a granular degeneration of the tubular cells, and the spleen considerable congestion, while the splenic pulp was largely drained of cells. In the marrow lymphoid tissue was replaced largely by fat.

Our experiments concern mainly the effect of intravenous injections of active deposit of radium and emanation on antibody formation, but since the toxic and other effects of radium on rabbits do not seem to have been studied, we made observations also on the changes produced in the organs and the leukocytes. Solutions of sodium chloride containing active deposit from radium emanation were used as described in detail by Bagg.³ The radium which served as the source of the deposit is the property of the Medical and Surgical Group of Denver and amounted to 496 mg., contained in a small glass flask in the form of a slightly acid solution of the bromide salt of the element. The emanation apparatus, the property of the Radium Company of Colorado, is a modified Debierne-Duane apparatus, designed by C. F. Whittemore, chief physicist of the company, to meet the Denver altitude requirements and differing in details and durability from the apparatus used by Duane⁵ at sea level. To remove the emanation from the solution and purify the gas, the entire apparatus was evacuated by an electrically operated oil-sealed, rotary vacuum pump, the mercury being so manipulated that the radium emanation, together with the hydrogen and oxygen, formed through decomposition of the water by the radium radiation within the flask, diffuses into a bulb whose volume is relatively greater than that of the flask containing the radium. These mixed gases are introduced into a series of chemical chambers in which the hydrogen and oxygen come in contact with an electrically heated, slightly oxidized copper wire and form water which is absorbed in the chamber containing phosphorus pentoxide. A second mercury pump, similar to the one used in drawing the gas from the radium flask, is used to collect the purified radium emanation from the chemical chambers and transfer it into a tube in which a few milligrams of pure sodium chloride has been fused. The purified radium emanation remains in contact with the fused sodium chloride for about 3 to 4 hours, when the maximum equilibrium amount of the active deposit of rapid change has been formed. This active

³ Bost. Med. & Surg. Jour., 1917, 177, p. 787.

deposit consists of radium A, radium B and radium C, and since radium C is the chief source of gamma radiation in any radium compound, this active deposit may be used for therapeutic purposes as long as the life of the active deposit permits. The life of this material is short, however, the gamma radiation from radium C decaying to one-half value in 60 minutes and to practically nothing at the end of 240 minutes. After the 3 to 4-hour period has elapsed, the radium emanation is drawn back into the purification apparatus and the bulb containing the active deposit on the fused sodium chloride is cut off and measured by the gamma radiation method. We usually obtained a measurement of from 120 to 230 millicuries. These measurements are made quickly to prevent loss by decay, and sterile distilled water added to dissolve the fused sodium chloride, the resulting solution containing a large portion of the radium active deposit (radium A, B and C). This is drawn into a syringe, which is then measured when containing the solution and after it has been emptied by comparison with the gamma radiation from a standard radium preparation; we obtained from 45 to 120 millicuries on the different days of preparation, which usually was 2 to 3 times a week. The instrument used was the usual gamma ray electroscope. The difference in the readings of the syringe plus solution and the empty syringe gives the approximate content of the solution that has been injected. The number of millicuries injected cannot be controlled exactly as an irregular quantity, from 20 to 50%, may remain in the syringe. The rapid decay of the active deposit introduces a second source of error. Therefore, the exact amount given can only be accurately estimated after all readings have been made.

In order to study the leukotoxic action and the changes produced by the active deposit in rabbits, graded intravenous injections, as nearly as these could be controlled, were given to a series of 13 rabbits ranging in weight from 2 to 3½ kilos. Total and differential counts were made of leukocytes before and at regular intervals after injection of the active deposit, and if the animal died the tissues were studied carefully. The results indicate that active deposit of radium emanation given intravenously in salt solution is lethal to rabbits in about 6 days in amounts exceeding approximately 20 millicuries measured by the gamma ray electroscope; an occasional animal may die from a smaller dose (16 millicuries), and larger amounts (48 millicuries) may prove fatal earlier. This form of radium produces first an increase in the circulating leukocytes as high as 34,600—as it did in a rabbit that died 4 days after the injection of 37 millicuries—which subsequently may

drop as low as 900, as it did in rabbit 3 just before death. In rabbits given a nonlethal dose the leukocytes may drop and gradually increase again, and with small amounts the counts are variable. The Arneth classification shows a shift to left with the onset of leukocytosis and then to the right as the leukocytes decrease and back to the left as a low count is reached. The mononuclear cells increase as the leukocytes fall, and in extreme cases there may be a marked relative lymphocytosis.

ANATOMIC CHANGES PRODUCED BY ACTIVE RADIUM DEPOSIT

Rabbit 1 (2.5 kilos).—Died 2 days after the intravenous injection of 48 millicuries. All veins of the skin and abdomen distended; abdominal muscles bright red; liver congested and friable, lobules well defined; spleen soft and blue; suprarenals large; kidneys pale and swollen; lungs congested, more in upper lobes and anterior; marrow of femur moist and pale. Microscopically the liver was intensely congested, the nuclei of the cells in many places pycnotic or disintegrated; the lymphoid tissue in the spleen appeared to be reduced and there was much cellular disintegration and many pigment containing cells in the splenic tissue, which was congested; the capillaries of the kidneys were dilated and there was blood in some of the collecting tubules; the epithelium of the tubules was granular, the nuclei pycnotic; the cells of the suprarenals were granular, the nuclei hyperchromatic and the vessels congested; there apparently was a decrease in the lymphoid cells in the mesenteric nodes; there were a few hemorrhages in the myocardium; the lungs were intensely congested and many of the alveoli contained blood and cellular detritus; the marrow of the femur was congested and the fatty tissues seemed to be increased, the cells diminished.

Rabbit 2 (3.1 kilos).—Died 3 days after the intravenous injection of 58 millicuries active deposit. The liver was pale and friable; spleen soft, small and purplish; kidneys pale, glomeruli congested; mesenteric lymph nodes swollen; lungs contained hemorrhagic foci, the left lung being solid anteriorly; marrow of femur pale and moist. Microscopically the liver was congested; the spleen congested, the lymphoid tissue reduced; the suprarenals congested, especially in the medulla; in the kidneys there was capillary congestion and blood in some of the tubules and glomerular capsules; there were many pigment containing cells in the mesenteric lymphoid nodes; the lungs were congested and there was blood in many bronchi and alveoli; in the marrow of the femur the cells were diminished and the fat tissue apparently increased.

Rabbit 3 (2.3 kilos).—Died 4 days after the intravenous injection of 37 millicuries active deposit. The spleen was purple and soft and the mesenteric nodes moist, otherwise the organs seemed to be normal. Microscopically there was congestion in most of the organs, the nuclei in the liver and suprarenal epithelium staining deeply; in the mesenteric lymphnodes and the marrow, the cells were reduced in number.

Rabbit 4 (2.3 kilos).—Died 5 days after the intravenous injection of 16 millicuries. The marrow of the femur was pale and much softer than normal; there was congestion in the liver, spleen, suprarenals, kidneys and lungs; in the spleen, mesenteric lymphnodes and marrow the cells seemed to be diminished in number and showed more or less disintegrative changes.

Rabbit 5 (2.5 kilos).—Died 6 days after the intravenous injection of 44 millicuries. The liver was congested and fatty; the spleen and mesenteric lymphnodes large and congested; the kidneys were also congested with a few

TABLE 1

THE LEUKOTOXIC ACTION OF INTRAVENOUS INJECTIONS OF ACTIVE DEPOSIT OF RADIUM EMANATION IN RABBITS

Amount of "Active Deposit", Given Intra- venously	Rab- bit	Time of Blood Examina- tion in Relation to "Active Deposit", Injection. Days Be- fore = B; After = A	Leukocytes on the Basis of 100 Cells Counted									
			Total per C.Mm.	Polymorphonuclears (Arneth Scale)					Eosino- phils	Baso- phils	Mono- nuclears	
				I	II	III	IV	V			Large	Small
37 milli- curies; fatal in 4 days	3 2.3 kilos	7 B	12,800	12	13	11	3	0	0	0	6	55
		1 B	16,700	8	13	10	1	0	0	0	4	64
		Same day	11,600	18	9	15	3	0	0	0	2	53
		1 A	34,600	52	33	10	0	0	0	0	1	4
		2 A	13,800	45	31	17	2	1	0	0	0	4
		3 A	1,000	12	13	4	0	0	0	0	7	64
		4 A	900*	1	17
44 milli- curies; fatal in 6 days	5 2.5 kilos	1 B	11,000	29	10	10	1	0	0	0	10	40
		Same day	30,000	20	24	10	1	0	0	0	6	39
		1 A	15,000	34	33	23	4	0	0	0	1	5
		2 A	8,300	14	18	15	2	1	0	0	2	48
		3 A	4,000	12	6	3	1	0	0	0	11	67
		4 A	3,000	6	7	2	2	0	0	0	19	66
		5 A	2,700	11	4	1	0	0	0	0	16	68
14.5 milli- curies; not fatal	8 3 kilos	6 A	2,800	6	15	5	0	0	0	0	7	67
		4 B	16,700	29	17	17	6	0	0	0	6	25
		1 B	15,500	12	20	20	4	2	0	0	9	33
		1 A	15,700	18	31	17	5	0	0	0	8	21
		2 A	5,000	8	23	21	11	3	0	0	4	30
		3 A	3,000	8	17	13	4	0	0	0	14	44
		4 A	2,900	10	17	17	3	0	0	0	9	47
		8 A	5,000	15	17	14	5	0	0	0	10	39
		10 A	5,000	7	13	18	1	1	0	0	12	48
		12 A	5,000	5	11	8	3	0	0	1	15	57
		14 A	6,000	13	11	8	2	0	0	0	16	50
		16 A	6,000	17	23	6	1	0	0	0	9	44
		18 A	16,000	11	11	8	1	0	0	0	12	57
20 A	13,000	11	15	7	0	0	0	1	20	46		
20 milli- curies; not fatal	11 2.7 kilos	3 B	21,400	26	9	3	0	0	0	0	8	54
		Same day	25,000	21	15	5	0	0	0	0	7	52
		1 A	19,000	32	29	8	2	0	0	0	13	16
		2 A	8,400	9	18	7	2	0	0	1	3	60
		3 A	3,000	7	5	3	1	0	0	0	12	72
		4 A	2,400	6	3	4	0	0	0	1	8	78
		5 A	3,000	21	6	0	0	0	0	0	5	68
		6 A	5,200	32	6	0	0	0	0	0	0	62
		7 A	3,600	26	7	0	0	0	2	4	5	52
		9 A	4,500	21	14	0	0	0	1	0	10	54
		11 A	11,000	27	6	2	0	0	0	1	4	60

* Too few cells.

Four typical examples only are given for purpose of illustration.

LYSIN AND PRECIPITIN FORMATION BY RABBITS GIVEN INTRAVENOUS INJECTIONS OF ACTIVE DEPOSIT OF RADIUM EMANATION

Treatment		Rabbits		Titers and Total Leukocytes in Relation to Antigen Injection																			
Time	Dose of Active Deposit in Milli-curies	Rabbits		Days Before Intraperitoneal Injection of Sheep Blood				Days After Intraperitoneal Injection of Sheep Blood															
		Number	Weight in Kilos	4	1	0	1	3	5	7	9	11	13	15	17	19	22	25	28	31	34	37	40
4 days before injection of sheep blood	10	1	4	(10.2)	(1.8)	24	0	0	192	1536	1536	768	192	384	768	768	768	1536	1536	1536	1536	1536	1536
						(8.6)	(8.0)	(10.4)	(5.4)	(4.6)	(12.0)	(8.2)	(12.0)	(9.8)	(10.6)	(10.8)	(9.6)	(10.2)	(9.6)	(15.0)	(16.4)	
4 days before injection of sheep blood	5	5	3.5	(13.0)	(54.0)	0	0	24	384	1536	3072	1536	768	768	768	768	768	1536	1536	1536	1536	1536	768
						(4.6)	(17.0)	(8.8)	(7.2)	(14.0)	(20.4)	(17.0)	(21.0)	(19.0)	(16.2)
4 days before injection of sheep blood	1	7	2.5	(15.2)	(8.0)	24	0	48	24	1536	3072	1536	1536	1536	1536	1536	1536	1536	1536	1536	1536	1536	Dead
						(10.0)	(14.0)	(16.4)	(23.0)	(12.4)	(14.4)	(11.2)	(23.0)	(12.0)	(12.2)	(5.8)
Coincident with sheep blood	10	12	3.5	24	0	24	768	768	6144	6144	3072	1536	1536	1536	1536	1536	1536	1536	1536	1536	1536
						(14.0)	(7.6)	(3.0)	(4.8)	(5.0)	(8.0)	(10.2)	(14.5)	1600	1600	1600	1600	1600	1600	1600	1600	1600
Coincident with sheep blood	5	13	3.3	0	48	0	3072	3072	6144	6144	3072	1536	1536	1536	768	768	768	768	768	768	168
						(10.0)	(16.0)	(7.6)	(7.2)	1000	3200	6400	3200	6400	3200	6400	3200	6400	3200	6400	3200	6400
Coincident with sheep blood	1	17	2.5	0	24	0	0	0	168	384	192	0	0	0	384	0
						(9.6)	(26.0)	(4.2)	(16.0)	(7.6)	(10.6)	(7.4)
5 days after injection of sheep blood	10	20	3.5	0	0	0	192	768	768	192	96	0	192	192	348	768
						(12.0)	(8.0)	800	800	3200	1600	1600	800	800	800	800	800	800	400	400
5 days after injection of sheep blood	5	23	3	0	0	96	96	1536	768	384	0	0	0	0	384	768
						(15.4)	(12.6)	(17.0)	(23.0)	(20.0)	(15.2)	(8.6)	(13.8)	(17.4)
5 days after injection of sheep blood	1	25	3	0	0	384	768	6144	3072	1536	384	384	96	0	0	364
						(14.0)	(10.6)	(8.0)	(6.6)	(11.2)	(9.4)	(10.4)	1600	1600	1600	1600	1600	1600	1600	1600
Controls	..	28	2.5	0	0	0	12288	12288	24000	6144	6144	6144	768	3072	1536
						(12.0)	(10.2)	(15.8)	(11.2)	(9.4)	(12.4)	(14.6)
Controls	..	30	2.7	24	0	0	6144	12288	6144	1536	768	384	24	384	1536
						(10.8)	(12.4)	(17.9)	(27.4)	(19.8)	(19.4)	3200	6400	6400	6400	6400	6400	6400	6400	6400

Leukocyte counts (in parentheses) are given in fractions of thousands, i. e., 10.2 equals 10,200 leukocytes per cmm. of blood; the figures above the leukocyte counts give the lysin titers, while the figures below the leukocyte counts give the precipitin titers.

small hemorrhages. Microscopically, besides congestion the liver showed granular changes in the cells; the splenic sinuses were filled with pigment containing cells; the tubules of the kidneys contained blood in places and the epithelial cells were loosened; the heart muscles showed congestion and loss of striation, many nuclei being pycnotic; the marrow of the femur was congested, the marrow cells diminished in number.

Rabbit 6 (3.2 kilos).—Died 6 days after the intravenous injection of 39 millicuries. The axillary, inguinal and mesenteric nodes were hemorrhagic with loss of lymphoid cells; the liver pale, cells granular, nuclei hyperchromatic; the kidneys showed punctate hemorrhages with granular changes in the tubular cells; the spleen was small, there was yellow pigment in the trabeculae and many cells appeared to be undergoing disintegration; the myocardium contained small hemorrhages and there was loss of striation; in the lungs were hemorrhagic bronchopneumonic foci; the marrow of the femur was congested and the cells greatly diminished in number.

Rabbit 7 (3.2 kilos).—Died 6 days after the intravenous injection of 26 millicuries. The liver was friable, congested, nuclei large, protoplasm granular; the spleen was small and contained brownish yellow pigment; the kidneys were congested and there were detritus and casts in the tubules; the heart muscle showed diminished striation; the lungs extravasation of blood in the alveoli; the marrow of the femur and the lymphnodes loss of cells and there were hemorrhages in the marrow.

The remaining rabbits (8-13) in this series lived after receiving from 1 to 20 millicuries.

The most important changes appear to be congestion and small hemorrhages in the lungs, kidneys, and other organs with disintegrative changes and loss of cells in the spleen, marrow and lymphnodes. On the whole, the changes are not unlike those found by Bagg in the white rat and dog after the injection of active deposit.

THE EFFECT ON ANTIBODY FORMATION

In order to determine the effect of active deposit on antibody formation 30 rabbits, from 2.5 to 4 kilos in weight, were given intravenous injections of approximately 1, 5 and 10 millicuries, 4 days before, coincident with or 5 days after the intraperitoneal injection of about 10 c.c. of citrated sheep blood per kilo of weight, and the specific precipitin and lysin for sheep blood determined at regular intervals afterward. The technic for estimating the antibody content of the serum was the same as in the previous experiments of similar nature.¹ The results, which are illustrated in table 2, show that active deposit of radium emanation in doses of 1, 5 and 10 millicuries intravenously may cause a depression in the formation of the specific lysin which may be quite marked in individual rabbits. The effect seems more evident when the active deposit was injected 4 days before or coincident with the antigen rather than when injected 5 days after. Table 2 shows that

the active deposit of radium emanation in toxic nonlethal doses also has some depressant action on the formation of specific precipitin for sheep protein no matter whether given before, coincident with, or after the injection of the antigen. The action, however, is not regular. This seems to go hand in hand with the rapid disintegration chemically of the active deposit which has a rather indefinite lethal dose, being lethal at times within wide ranges for rabbits when given intravenously.

SUMMARY

Active deposit of radium is lethal to rabbits when given intravenously in amounts of approximately 8 to 10 millicuries per kilogram weight. In lethal amounts given intravenously the active deposit produces an initial leukocytosis with finally a marked diminution in the circulating leukocytes, mainly of the polymorphonuclears. This is associated with changes in the liver, lungs, lymph glands, spleen, suprarenals and kidneys frequently accompanied by capillary hemorrhages. These results are similar to those obtained with active deposit in white rats and dogs by Bagg. Given intravenously, active deposit in non-lethal amounts may have a depressing effect on the formation of lysin and to a less extent on precipitin for sheep blood.

SOME ANTIGENIC RELATIONS OF THE BIPOLARIS SEPTICUS GROUP OF BACTERIA *

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INTRODUCTION

Diseases of various animals, including the horse, ox, sheep, hog, fowl, and rabbit, have been described and the etiologic rôle in such conditions attributed to bacteria of the bipolaris septicus group. While these organisms are found under diverse conditions and in animals affected with what is commonly termed hemorrhagic septicemia, their pathogenicity is yet by no means clear, except perhaps in fowl cholera. The symptoms, and particularly the lesions of the disease, show a remarkable similarity in various hosts. The bacteria isolated therefrom agree in their morphologic features and cultural characteristics. The possibility exists that there is only a single organism, although there is little evidence to show that the various diseases are intertransmissible from one species of animal host to another, although the contagiousness of the conditions for some animals certainly is slight. The question arises, How pathogenic are these bacteria for their homologous hosts under natural conditions? That phase of the problem awaits further study lest too much be assumed without exact substantiation.

This study was undertaken for the purpose of making an immunologic comparison of various strains of the bipolaris organism by means of the complement-fixation reaction, and using the serum of hyperimmunized animals. If there is a true host specificity or a specific pathogenicity in animals, it might be ascertained from such studies. If there is an inherent difference in the bacterial protoplasm, it may be assumed that the antibodies resulting from the antigenic property of these bacteria may furnish means of differentiation. If several distinct organisms occur, it may be possible to demonstrate type or variety by means of the blood serum of animals naturally infected. This does not appear feasible since even routine immunologic methods appear impracticable in diagnosis of these conditions. Obviously the questions of unanimity of type or of numerous disease strains, as many as are

Received for publication, May 29, 1922.

* These studies were instigated while the writer was on leave of absence and permitted to work in the Pathological Division of the Bureau of Animal Industry, at Washington, during the winter of 1920. The experimental work was subsequently done at the North Dakota Agricultural College.

susceptible hosts, have been debated, as will appear from a brief summary of available literature relative to both pathogenic and immunologic relationships of this group of bacteria.

HISTORICAL

Bollinger¹ and others demonstrated the transmissibility and pathogenicity for rabbits, sheep and goats of material from animals which had died of wild-und-rinderseuche. Pease² observed an infection in Indian buffaloes which was transmissible not only to buffaloes but likewise to cattle, horses, pigs, guinea-pigs, rabbits, mice and pigeons. The bacteria were identified as those of rinderseuche. Holmes³ quotes Chamberland and Jouan as denying the specificity of *Pasteurella* for each species and contending that all forms of pasteurellosis are produced by the same microbe, more or less virulent and more or less adapted to different species of animals. Kitt⁴ states that fowl cholera was to be reproduced in none of the larger domesticated animals through feeding. Joest⁵ finds that the *suisepcticus* is apparently as pathogenic for cattle, horses, sheep and goats as for hogs if injected intravenously.

Beck and Koske⁶ showed that a transmission of *suisepcticus* through ingestion with the feed is possible in sparrows, crows, pigeons, chickens and geese. Ducks could not be infected. Koske states that the ease of transmission of the *schweinepest* to those types of birds through feeding is worthy of notice since feeding experiments with fowl cholera do not always lead to general infection. Furthermore, neither *avisepticus* nor *suisepcticus* serum could be relied on to establish by the agglutination reaction a differentiation between *suisepcticus* and *avisepticus*.

F. Hutyra⁷ asserts that with proportionate quantities of organisms almost any animal is to be infected by intravenous injection. He further reports that an immune serum produced with one variety furnishes active protection against the homologous strain, but the protective action fails or only partially succeeds against other varieties.

Hutyra and Marek⁸ report Wassermann and Ostertag as having observed that blood serum from animals which were highly immunized with cultures or extracts of *Bacillus suisepcticus* protect usually only against the same strains of this bacillus, whereas it is without effect against other strains. On the other hand, Klepzw⁹ asserts that the blood serum of an animal strongly immunized against one member of the hemorrhagic septicemia group can be utilized as a curative agent against all forms of the disease.

Citron and Pütz¹⁰ likewise assert that there is a close relationship between the various strains. Mori¹¹ found that an *ovisepticus* immune serum agglu-

¹ Wild und Rinderseuche welche in Sommer 1878 in der Umgebung von München beobachtet wurde, 1878.

² The Veterinarian, 1898, 71, p. 278.

³ Memoirs of the Dept. of Agriculture in India, 1914, 11, p. 105.

⁴ Kitt, Th., in Geflügelcholera, Kolle und Wassermann: Handbuch d. Path. Mikroorg., 1913, 6, p. 37.

⁵ Schweinepest u. Schweineseuche, 1906.

⁶ Arb. a. d. Kais. Gesundheitsamte, 1904-05, 22, p. 503.

⁷ Septicemia Hemorrhagica, in Kolle und Wassermann: Handbuch. d. path. Microorg., 1913, 6, p. 77.

⁸ Special Pathology and Therapeutics of the Diseases of Domestic Animals, 1912, p. 137.

⁹ Hygiene, 1903-04, 1, p. 221.

¹⁰ Ztschr. f. Hyg. u. Infektionskr., 1907, 56, p. 145.

¹¹ Extract, Experiment Station Record, 1918, 38, p. 887.

tinated the strains derived from other animals. Gallagher,¹² in his work observed that an avisepticus strain which was virulent conferred a fair degree of immunity in rabbits against some strains of *B. bovissepticus*. Such rabbits resisted an infection with a virulent strain of *B. suissepticus*.

Authorities certainly differ regarding the question of whether there is but one or a multiplicity of strains. Clinical reports concerning the simultaneous occurrence of infections caused by these bacteria are rare. Such might naturally be expected if the extreme virulence which has been assigned to them is not substantiated.

The epidemiology and predisposing factors for these diseases are so obscure that explanations are indeed difficult. Fowl cholera and possibly infections in sheep are most common. Authorities who are not actuated by commercial reasons are agreed that infections in the larger domestic animals are not of common occurrence. Mohler and Eichhorn¹³ make the general statement that hogs became affected with swine plague following an outbreak of hemorrhagic septicemia in cattle. Further, the disease in sheep has developed subsequently to an infection in hogs. However, in this connection an infection may possibly occur simultaneously in two or more animal species yet not involve the same particular strain of organism.

Matsuda¹⁴ studied by means of the complement-fixation test the relationship of the bipolaris bacteria to various immune serums prepared with different strains of the organism. He prepared vaccines by autolyzing the bacterial growth with distilled water and inactivating at 44 C. for 3 hours. It appears from the tables that a single injection brought about a reaction whereby the serum with an appropriate antigen readily bound the complement. The phenomenon was more marked if the injections were repeated. His data indicate that bovissepticus, suissepticus, and cuniculissepticus serums show a much higher degree of specific fixation with their homologous antigens than with heterologous bipolaris antigens. This apparently is a method whereby one might distinguish between the various strains of the group. Furthermore, it would appear that actual antigenic differences exist between the various animal strains which might or might not be represented by individual pathogenic characteristics.

CULTIVATION AND GROWTH

Cultures of those organisms included generally in the hemorrhagic septicemia group possess certain well defined characteristics. No others are considered in the studies included in this paper. A 3% glycerol agar with a reaction prior to sterilization of P_H 7.2 has given satisfactory results for cultivation. Such cultures are nonproteolytic in that they do not liquefy gelatin. No gas is formed from any carbohydrate although acid is produced from dextrose, sucrose, levulose, galactose and mannose. Lactose is not fermented, neither is any change produced in litmus milk. They are nonmotile, gram-negative and fail to grow on potato. The appearance of the growth in the broth of the fermentation tube shows the aerobic character of these

¹² Jour. Am. Vet. Med. Assn., 1917, 50, p. 708.

¹³ Am. Jour. Vet. Med., 1916, 8, p. 14.

¹⁴ Ztschr. f. Hyg. u. Infektionskr., 1916, 66, p. 383.

bacteria; the broth is perceptibly cloudy in the open arm, with growth slowly ascending in the closed arm with a clear line of demarcation marking the upper limit of growth. The bacteria are highly pathogenic for rabbits and may function as antigens in the complement-fixation test with a specific immune serum.

Besemer¹⁵ found the various strains to agree in their action on the various carbohydrates. He concludes that there seems to be no cultural or biochemical basis for designating by different names the five members of this group.

Bacteria which do not conform to these growth characteristics have been isolated from animals manifesting the symptoms and lesions designated as hemorrhagic septicemia. Such bacteria frequently have the characteristic bipolar staining property of the bipolaris group. Certain members of the paratyphoid, enteritidis group, *B. coli*, and *B. proteus*, may thus be easily mistaken for hemorrhagic septicemia bacteria. Some investigators assert that many organisms recently isolated from the animal body may show bipolar staining and yet possess few of the other cultural characteristics of the bipolaris group. Busson¹⁶ seems to include a great variety of bacteria as the causative agents of hemorrhagic septicemia. Their cultural reactions reveal little relationship however.

It is true that animal experimentation is a more definite method whereby the relative susceptibility of various animal species for these bacteria might be determined. If some strains have a well defined pathogenic effect on some species and not on others they might reasonably be expected to induce disease conditions in only their particular hosts. However, suisepticus and bovissepticus cultures are apparently incapable of regularly reproducing an infection in their respective hosts except such cultures be injected subcutaneously or intravenously. It is doubtful whether such bacteria are the primary cause of the infection or merely secondary invaders which take advantage of the lowered resistance on the part of the host. Perhaps in chickens the bipolaris organism plays a primary rôle, yet the widespread prevalence of fowl cholera in North Dakota during the winter and spring of 1921 associated with the isolation of farm flocks which there prevails, again compels one to consider the possibility of predisposing influences even in infections with avissepticus in domestic fowls.

¹⁵ Jour. Bacteriol., 1917, 2, p. 177.

¹⁶ Centralbl. f. Bakteriol., I, 1921, 86, p. 101.

IMMUNOLOGIC METHODS

Studies in the laboratories of the Pathological Division of the Bureau of Animal Industry of the Department of Agriculture have shown that commercial hyperimmune serums prepared for curative purposes against hemorrhagic septicemia will bind the complement in the presence of broth culture antigens of various members of the bipolaris group. The reaction is shown by all cultures which conform to the growth characteristics of this group. It matters not if the culture is a hemologous or a heterologous strain with which the serum was presumably prepared. If a culture of some other organism, e. g., paratyphoid, is used as a broth antigen with such a serum, no greater degree of specificity is shown by such a serum than the anticomplementary point as given by a normal horse serum. It must be said, however, that some commercial hemorrhagic septicemia serums show evidence that foreign bacteria have been used in their preparation. Obviously such serums would probably be unsatisfactory for identification of bipolaris cultures.

A commercial suissepticus serum (0.0055) was used for controlling the density and specificity of culture antigens. Little quantitative specificity was to be observed with this serum. As 0.05-0.1 c c of serum is required to fix a quantity of broth antigen, it matters not whether a suissepticus or any heterologous strain is employed.

The question now arises, Were only cultures of one animal host variety employed in the preparation of this and similar serums which have given like results in the Bureau laboratories? Doubtless the manufacturers of such products employ cultures from many sources. Were all cultures for this serum derived from hogs? If they were, why do cultures from cattle, sheep, fowls, guinea-pigs and rabbits bind the complement and function as antigens in the same manner as suissepticus cultures? There are two probable explanations; one of them is that there is only a single organism—a view which is held by many. The other is that several varieties or types were used either knowingly or unintentionally in the preparation of commercial hyperimmune serums which are doubtless polyvalent.

As Matsuda worked with univalent serum, it was considered advisable to prepare serum from animals, using single cultures in order either to verify his findings or to confirm the general conclusions reached by the Bureau.

The experimental phases of this work deal, then, with the preparation of a number of specific immune serums from rabbits in which only

a single culture strain was used on individual rabbits, and the testing for specificity of these serums against cultures isolated from various hosts by means of the complement-fixation test. It was suspected that such work might throw further light on the problem of whether one or many organisms infect different animals.

SOURCE OF CULTURES

It was necessary to work with cultures from well authenticated sources, otherwise misleading results might be obtained. Care was therefore exercised to secure cultures from laboratories in which they were originally isolated, or from investigators who could vouch for the species host of origin.

Cultures of the following strains were obtained from the Pathological Division of the Bureau of Animal Industry, United States Department of Agriculture: bovisепticus cultures K. C. 298, Nos. 67 and 28, suisепticus cultures No. 64, K. C. Nos. 8 and 9; ovisепticus cultures 56, 52 and 47, and avisепticus cultures No. 20 and Colorado 28, cavia 65 and the rabbit strain.

The following were obtained from the Pathological Laboratory of the Colorado Agricultural College: ovisепticus Colorado 33, avisепticus Colorado 15 and suisепticus Colorado 13.

The following were obtained from the Veterinary Department of the Kentucky Agricultural College: suisепticus cultures designated Ky. 2, 3 and 4 and avisепticus Ky. 6.

The following were isolated by the writer from the corresponding postmortem material: bovisепticus 6070, avisепticus 58 and 59 and goose 6274.

PREPARATION OF SPECIFIC SERUM

The process of preparation of the various serums from rabbits and the one horse so utilized was essentially the same throughout the work. Rabbits were bled prior to commencement of the immunization process. The normal serum so obtained was preserved for subsequent control. The normal serum in a quantity of 0.05 cc of no rabbit was found to produce appreciable fixation with the antigen subsequently used in its immunization. Vaccines were prepared from such cultures as were desired to be used in process of immunization. Care was exercised to use only one culture from a well authenticated animal origin on each group of rabbits throughout the process of immunization. Furthermore, necessary precautions were employed to insure freedom from contamination in such cultures. The cultures were grown for 24 hours on large agar slants containing 3% glycerol. The growth was then removed and suspended in salt solution. No attempt was made to standardize such bacterial suspensions, but they were made preceptibly milky so that a rather heavy suspension was always employed. The suspensions were heated for 1 hour at 56 C.

The rabbits received from 4 to 6, 2 cc doses of the appropriate vaccine. There was little difficulty in producing a considerable degree

of immunity in rabbits by such a process. That is, rabbits which received 5 subcutaneous injections at intervals of about 4 days of 2 cc doses of a heavy, killed suspension of a hemorrhagic septicemia culture, later sustained without difficulty doses of living cultures which in like amounts killed untreated rabbits within 24 hours.

Live organisms were then employed to advance further the process of immunization. Small doses were given subcutaneously at first, then cautiously larger amounts were given and at times intravenous injections were attempted, but they frequently resulted disastrously, although large subcutaneous injections of live organisms were readily tolerated without fatal termination. As serum with a high antibody content was desired, care was exercised to prolong the process of immunization until preliminary tests with the blood showed perceptible amounts of complement-fixing antibodies for the homologous culture of bipolaris septicus. When it was found that the serum had attained considerable specificity for the antigen employed, the rabbits were bled and the serum preserved.

It hardly appears necessary to submit the schedules of immunization of all the rabbits employed in the preparation of serum. The character of the materials injected and the general scheme of injections were similar for the different series of rabbits. However, the following schedule is representative of the methods employed:

PREPARATION OF SERUM WITH OVISEPTICUS 56

Rabbits 13, 15, 16:

- 7-1-20 2 cc of the corresponding vaccin, subcutaneously.
- 7-6-20 2 cc of the corresponding vaccin, subcutaneously.
- 7-12-20 2 cc of the corresponding vaccin, subcutaneously.
- 7-14-20 2 cc of the corresponding vaccin, subcutaneously.
- 7-17-20 2 cc of the corresponding vaccin, subcutaneously.
- 7-22-20 1 cc of a suspension of live organisms subcutaneously.
- 7-26-20 1 cc of a suspension of live organisms intravenously.
- 7-28-20 Rabbit 15 dead.
- 8-7-20 No. 13, 2 cc of a suspension of live organisms subcutaneously.
- 8-12-20 No. 13, 2 cc of a suspension of live organisms subcutaneously.
- 8-19-20 No. 13, 2 cc of a suspension of live organisms subcutaneously.
- 8-23-20 No. 13, 1 cc doses of live organisms subcutaneously.
- 8-30-20 No. 13, 1 cc doses of live organisms subcutaneously.
- 9-4-20 No. 13, 1 cc doses of live organisms subcutaneously.
- 9-7-20 No. 13, 1 cc doses of live organisms subcutaneously.
- 9-15-20 No. 13 bled for serum.

No especial attempt was made to determine the relative immunity of the rabbits at any stage of their immunization, although certain observations were possible. Neither was any attempt made to ascertain whether a specific immunity for only one strain had been developed; that is, are rabbits immunized to one strain likewise immune to strains of heterologous animal origin? One of the significant facts observed is the rate of development of immune bodies, or rather the rate at

which complement-fixing antibodies are formed. I am not prepared to assert that such substances are identical or that the processes are parallel. This observation applies both to the rabbits and to the preparation of the immune serum from horse 6220. It was found on preliminary tests that even though an animal showed considerable immunity, no appreciable amounts of complement-fixing substances could be demonstrated. It was necessary to give further repeated injections, and, in order to obtain the maximum antigenic property, live organisms were employed before that animal's serum was suitable for the purposes of this experiment. The rate of development of a condition of immunity observed in rabbits substantiates the findings of Van Es and Martin¹⁷ and of Graham and Schwarze.¹⁸

Perhaps these observations have some practical applications and relationships. Since an animal responds so slowly to a stimulation with either an infection or to the process of immunization, as shown by the presence of immune bodies which can be demonstrated *in vitro*, there is small wonder that in an acute disease process induced by such organisms no substance of diagnostic value which can be demonstrated is as yet present. Likewise the question also arises, will small amounts of such vaccins as are commonly used induce sufficient immunity to protect an animal against field infection? It appears not, if appreciable amounts of virulent material are incorporated in the body of an animal with a high degree of susceptibility.

QUANTITATIVE TITRATION OF SPECIFIC IMMUNE SERUMS

It hardly appears necessary to outline all the routine steps in the performance of the complement-fixation reaction as applied to this work. Most of those details are in evidence from a study of table 1. The same methods were used throughout the work.

The preparation and titration of the individual antigens were the essential steps in the work. Broth cultures of the various strains were employed as antigens, and usually glucose broth fermentation tubes which were inoculated and incubated from 24 to 48 hours were utilized. Only such fresh preparations are suitable. Such cultures are diluted with plain broth until the solution is opalescent rather than cloudy. Preliminary titration of such antigens with a known positive hemorrhagic septicemia serum (0.0055) was always regularly performed. The antigenic point was thereby determined before it was

¹⁷ Nebraska Experiment Station Research Bulletins 17 and 18, 1920.

¹⁸ Jour. Am. Vet. Med. Assn., 1921, 59, p. 546.

TABLE 1
TITRATION OF ANTISERUMS

No. of Tube	C c of 0.85% Salt Solution	C c of Antigen	Complement,* C c	Serum 0.0055 in C c		Amboceptor,† C c	Sheep Cells, 3% Suspension, C c		Results
1	1.5	0.05	1.0	0.2	Incubation for 1 hr. at 37 C.	1	1	Incubation for 1 hr. at 37 C.	0
2	1.5	0.1	1.0	0.2		1	1		+
3	1.5	0.2	1.0	0.2		1	1		++
4	1.5	0.3	1.0	0.2		1	1		+++
5	1.5	0.4	1.0	0.2		1	1		+++
6	1.5	0.5	1.0	0.2		1	1		+++
7	1.5	0.6	1.0	0.2		1	1		+++
8	1.5	0.8	1.0	0.2		1	1		+++
9	1.5	1.0	1.0	0.2		1	1		+++
10	1.5	2.0	1.0	0.2		1	1		+++
				Normal horse serum					
1	1.5	0.05	1.0	0.2	Incubation for 1 hr. at 37 C.	1	1	Incubation for 1 hr. at 37 C.	0
2	1.5	0.1	1.0	0.2		1	1		0
3	1.5	0.2	1.0	0.2		1	1		0
4	1.5	0.3	1.0	0.2		1	1		0
5	1.5	0.4	1.0	0.2		1	1		0
6	1.5	0.5	1.0	0.2		1	1		0
7	1.5	0.6	1.0	0.2		1	1		0
8	1.5	0.8	1.0	0.2		1	1		0
9	1.5	1.0	1.0	0.2		1	1		0
10	1.5	2.0	1.0	0.2		1	1		+

* Fresh guinea-pig serum so diluted that each c c contains $1\frac{1}{2}$ units of complement as determined by previous titration.

† Sheep-rabbit hemolytic amboceptor diluted so that each c c contains $2\frac{1}{2}$ units.

0 denotes complete hemolysis with no fixation of complement; + - +++ denotes varying degrees of deviation of complement.

TABLE 2
TABLE OF AVERAGE AMOUNTS OF SERUM REQUIRED FOR FIXATION

Rabbit Serum	Culture Antigens			
	Ovissepticus	Avissepticus	Suissepticus	Bovissepticus
Ovissepticus* No. 6645	2 av. 0.001 c c	2 av. 0.0015 c c	2 av. 0.09 c c	1 required 0.1 c c
13	4 av. 0.0085	5 av. 0.01	5 av. 0.077	3 av. 0.077
32	5 av. 0.011	5 av. 0.008	6 av. 0.025	3 av. 0.04
Avissepticus No. 34	5 av. 0.013	5 av. 0.013	5 av. 0.044	3 av. 0.04
6 and 8	4 av. 0.005	6 av. 0.005	5 av. 0.044	3 av. 0.066
	43 titrations average 0.0087 c c		33 average 0.054 c c	
Suissepticus No. 1	4 av. 0.1 c c	5 av. 0.1	6 av. 0.017	3 av. 0.009
28	4 av. 0.042	5 av. 0.06	5 av. 0.013	4 av. 0.009
6220	3 av. 0.13	3 av. 0.12	4 av. 0.03	2 av. 0.05
Bovissepticus No. 1	4 av. 0.08	5 av. 0.07	6 av. 0.014	3 av. 0.02
23	5 av. 0.06	5 av. 0.1	6 av. 0.028	3 av. 0.02
	43 average 0.083 c c		42 average 0.018 c c	

* Refers to type of culture used in immunization of rabbit. Number designates rabbit serum.

possible to ascertain the relative potency of the specific rabbit serum against individual cultures.

It will be observed from table 1, which is representative of all such preliminary titrations, that the titration is made with an increasing quantity of antigen against a constant quantity of suisepticus serum (0.0055). It will be noted in this illustration that 0.3 c c of antigen was sufficient to produce fixation. With a negative horse serum not even 1 c c of the antigen was anticomplementary. Thus there is a high degree of specificity for the particular culture antigen titrated. When employed for quantitative titration against the rabbit serums 2 to 6 times (usually 3 times) the antigenic dose as shown by the preliminary titration was tested as a constant against decreasing dilutions of rabbit serums. Yet on the other hand, the test quantity of antigen should not approach the anticomplementary quantity.

DISCUSSION

The experimental work of this problem involved the testing of each serum against a number of homologous culture antigens and likewise against a variety of heterologous cultures. Serums were prepared from 2 cultures respectively of bovissepticus, suisepticus, ovissepticus, and avissepticus, and 1 of rabbit septicemia. Even casual examination of the records which necessarily are too cumbersome for publication shows that there is little to indicate that there are as many different strains as there are animal hosts. This differs markedly from the findings of Matsuda; on the other hand, it is quite evident that there was a wide difference in the amounts of a given serum necessary to produce fixation with the different culture antigens, other factors being uniform in each instance similar to the method followed in the Bureau laboratories.

A serum was obtained in the preliminary work from rabbit 6645, immunized with ovissepticus 56, which proved to be of comparatively high specificity and potency. This is particularly applicable when it is appreciated how difficult it is to induce the formation of immunity reaction producing substances with these organisms. Careful and repeated trials with different cultures and under a variety of conditions indicated that about 0.001 c c of such serum was sufficient to produce complete fixation with ovissepticus and avissepticus cultures but that even 0.05 c c gave little perceptible fixation with bovissepticus and suisepticus cultures. Such results being obtained, it was decided to prepare a series of serums as previously mentioned for further study and to determine whether similar specific relations would likewise exist.

BIOMETRICAL ANALYSIS OF DATA

The data obtained can be better analyzed, not by a numerical tabulation of individual titrations, but rather by obtaining the average quantity of a serum which was just sufficient to produce fixation with the various culture antigens. This method of analysis has been selected since the results obtained are similar to those shown by the plotting of curves using the quantity of serum used in the tests as ordinates. The averages tabulated herewith agree with the peak of the superimposed curves secured by the graphic method of analysis. Serums prepared with cultures of sheep and fowl origin gave reactions which indicated no difference in antigenic character. Cultures of rabbit and of cavia origin gave results similar to those of fowl and sheep. Similarly, cultures of porcine and bovine origin are grouped together.

Analysis of the data shows that of the titrations the average amount of ovisepticus and avisepticus serum necessary to produce fixation with 36 titrations with ovisepticus and suisepticus cultures was 0.054 c c but with 43 titrations with ovisepticus and avisepticus cultures it was but 0.0087 c c.

Similarly with bovisiepticus and suisepticus serums 43 titrations were made with ovisepticus and avisepticus cultures and 42 with the homologous group. Such serums required 0.018 c c to produce fixation with the homologous cultures, but with ovisepticus and avisepticus strains 0.083 c c was required.

As this problem originated in the Bureau laboratories, the Bureau requested permission to check the accuracy of the work done. Such serum as was available and cultures were furnished accordingly, and an analysis of the data so obtained reveals the fact that practically parallel results were there obtained. However, as a different proportion of titrations was made with some serums, using with some only a few cultures, than was here performed, the comparison is not exact. Further, the data of the Bureau includes figures with weak and comparatively impotent serums not employed by the writer. The work with ovisepticus and avisepticus serum show with 35 titrations with ovisepticus and avisepticus cultures 0.0197 c c was sufficient to produce fixation, while with 35 with bovisiepticus and suisepticus cultures 0.0475 c c was required to produce fixation. Similarly with bovisiepticus and suisepticus serums, 44 titrations with ovisepticus and avisepticus cultures 0.046 c c was required to produce fixation, while with 38 titrations with bovisiepticus and suisepticus serums only 0.0308 c c was necessary to produce fixation.

It appears, therefore, that serum prepared with ovisepticus and avisepticus cultures will fix the competent in appreciably smaller quantities with avisepticus and ovisepticus than with either bovisiepticus or suisiepticus culture antigens. A similar relationship, although the specificity is not so great, exists between bovisiepticus and suisiepticus serums and the homologous and heterologous serums. Potent serums show a much greater tendency to differentiate cultures in this manner than do weak serums, e. g., serum 6645.

There appears to be only 2 consistent exceptions to the foregoing groupings. Bovisepticus culture 6070 gave a much higher degree of specific fixation with ovisepticus and avisepticus serums than with bovisiepticus or suisiepticus serum. Likewise several titrations with avisepticus Ky. 6 appeared to group that organism with the bovine-swine type rather than with the ovine-avian group. Those titrations are therefore omitted from a statistical study of the data of this laboratory.

A series of tests were also made using constant quantities of a given serum in tests against increasing quantities of broth antigens (0.05—2 c c). The serum which was used in amounts of 0.02 c c to eliminate better the group complement-fixing substances, was sufficient to bind the complement when only small quantities of culture antigens of the homologous group corresponding to the serum was used. When a heterologous culture was used specific fixation was absent. Those results further substantiate the previous data.

The serum of an animal immunized to either avisepticus or ovisepticus shows some ability to produce fixation with suisiepticus or bovisiepticus antigens, although it is less than for the homologous type antigen. This is explained on the basis of a group relationship and the formation of group complement-fixing substances. Specific complement-fixing substances are quite evidently present, so that it appears that there is not a single organism.

These antigenic relationships may be explained on the basis of two types of bacteria, a bovine-swine and an ovine-avian rabbit-cavia type. Whether these are 2 distinct organisms with reference to their pathogenic properties is an open question. This antigenic difference is apparently sufficient to justify the assumption that there is a difference in the specific protein composition of the 2 groups or types. That an infection may occur simultaneously in sheep and fowls, or be inter-transmissible, is not impossible. A difference in antigenic property was also noted between fowl-sheep cultures and hog-bovine strains

inasmuch as rabbits responded to immunization with avian and ovine strains and thereby developed more potent serums than the same corresponding treatment with swine and bovine strains. It was also noted that cultures isolated from fowls and sheep showed a considerably greater specificity when their antigenic titer was measured in titrating against serum 0.0055 than did hog-cattle strains in which the anticomplementary point was less far removed from the antigenic quantity. Those observations may be interpreted as meaning that the avian-ovine type is more pathogenic while the bovine-swine variety is more saprophytic. Routine diagnostic work has convinced me that fatal septicemic infections with this group of bacteria are so rare, except in fowls and occasionally in sheep, that type determination would be of no significance. It has been the purpose in this paper to show, however, that there is an appreciable antigenic specificity revealed by the reactions invoked in animals by these bacteria. Certainly a higher degree of immunity might be expected by immunization in fowls and sheep than in hogs and cattle from the same process. Furthermore, if control methods of these diseases involve immunization, it appears that a product prepared from one or more cultures of a given type would give satisfactory results on all homologous animal types.

CONCLUSIONS

Rabbits and a horse injected with bacterial material from bipolaris septicus cultures developed complement-fixing substances for the respective antigens. The rate of development of such substances was slow.

It appears from these studies that the reaction of animals to the process of immunization with these organisms is slow. This is revealed both by the rate of development of substances which can be demonstrated by the complement-fixation reaction and by the relative degree of resistance which is induced in the animals against a homologous infection. Such conclusions must necessarily reflect on the efficiency of current methods of immunization wherein an appreciable immunity is desired. These conclusions agree with those of investigators published elsewhere.

A series of complement-fixation tests between rabbit serums prepared by such methods and various cultures gave results which indicate that there are differences in the specific protein composition of such bacteria as revealed by their antigenic properties which may be explained by assuming that there are two types of the bipolaris septicus bacteria, a bovine-swine type and an ovine-avian-rabbit-cavia strain.

THE ACID PRODUCTION OF BACILLUS WELCHII

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The studies recorded herein confirm and extend our previous observations with *B. welchii*,¹ but, in part, yield new interpretations.

We had observed that *B. welchii*, grown in a meat peptone mash medium, gives rise to an increased $H +$ ion concentration culminating at about P_H 6.1 in from 12 to 24 hours, followed by a gradual decline toward neutrality (P_H 7) in about 200 hours. We ascribed the increase in $H +$ ion concentration to the acid produced in the fermentation of the muscle sugar, and the decline in the curve to the neutralization of this acid by ammonia, presumably set free from the protein in the effort of the micro-organism to supply its carbon requirements. Kendall, Day and Walker² have since shown that *B. welchii* produces several times as much ammonia in mediums not containing fermentable sugars as in those containing them. In this respect the metabolism of this and other obligate anaerobes resembles that of the aerobes.

Corresponding to the increase in $H +$ ion concentration there was also an increase in titratable acidity, followed, during the period of continued depression in $H +$ ion concentration, by a slight decline, with a secondary, and usually greater, increase in titratable acidity, also followed by a fall. No such secondary peak occurred in mediums from which the meat particles had been filtered, and so we referred to the first peak as the "sugar phase" and to the second as the "protein phase."

Having secured these results in mediums to which no carbohydrates had been added, since we were then interested mainly in the elimination of muscle sugar in the preparation of "sugar-free" mediums, we now undertook to determine the effect of certain added fermentable sugars on the $H +$ ion concentration and titratable acidity curves, and their interpretation with particular reference to the utility of the peaks of these curves as criteria of freedom from sugar.

Received for publication, June 6, 1922.

¹ Jour. Infect. Dis., 1921, 29, p. 344.

² Ibid., 1922, 30, p. 141.

EXPERIMENTS

Our general methods have been described.¹ The mediums now to be mentioned were made, however, with "Difco" peptone instead of that of Parke, Davis & Co., and 1 liter flasks containing 900 c c of medium were used instead of the smaller sizes to avoid any considerable alteration in volume through sampling.

The meat mash mediums were prepared by the addition of the meat residue, removed from the meat infusion by straining and filtration after boiling, to the clear 2% peptone meat infusion broth in the proportion of 100 gm. of meat per 800 c c of broth. Sugar mediums were resterilized on 3 successive days for 20 minutes daily in the Arnold sterilizer.

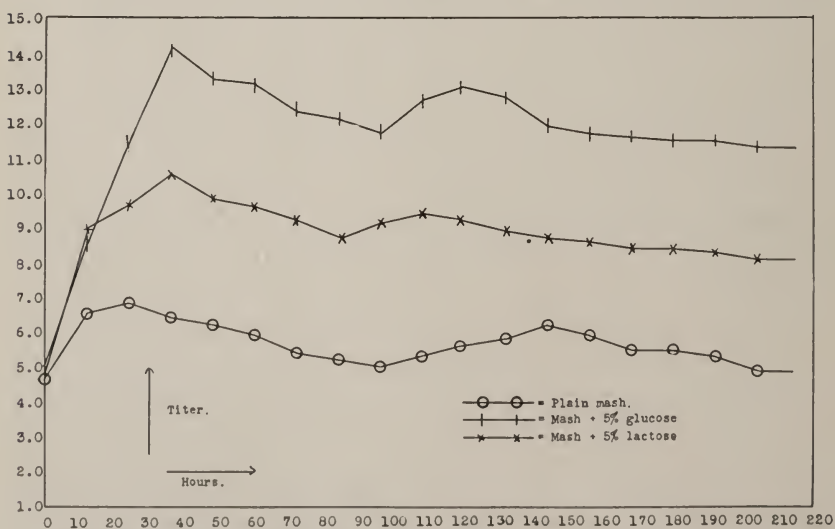
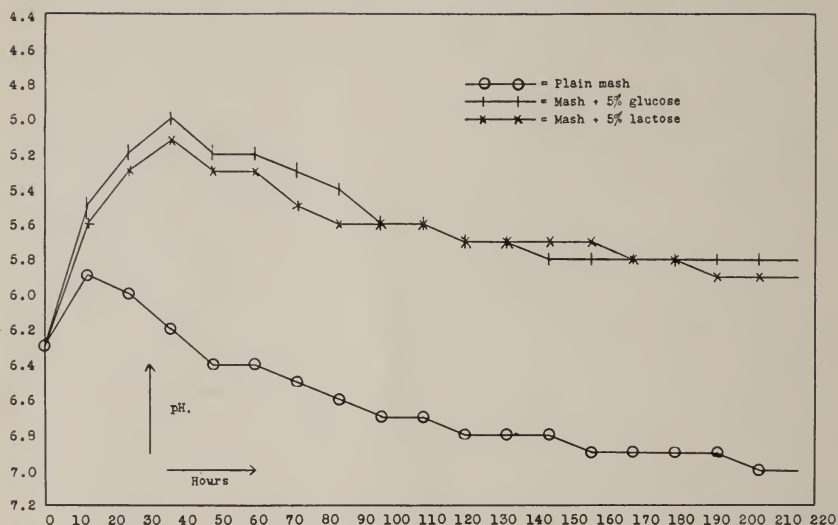
Sampling, titration, determination of H^+ ion concentration, and the usual check tests for purity, were as before, and the same culture of *B. welchii* (No. 2, for description and origin of which see Hall³) was used. Our data were analyzed and are presented as before by the graphic method; the charts are self-explanatory, H^+ ion concentration being shown in terms of P_H values and the corresponding titers in number of c c N/20NaOH required to neutralize 5 c c of a given sample to phenolphthalein, i. e., percentage normality.

Our first experiment was to duplicate the peculiar results with meat peptone mash medium under the slightly changed conditions of experimentation (peptone and larger flasks). There was no difficulty in this; the results practically coincided with those formerly reported¹ (charts 7 and 8). A similar test was included as a control in each of the experiments herein reported.

We then studied the effect of an excess (5%) of added glucose and lactose on the H^+ ion concentration and titratable acidity, as shown in chart 1.

It was no surprise to note in the plotted data the low P_H values of the sugar mediums as compared with those of the plain mash, but that there should be a peak in the curves for the former similar to that of the latter was not anticipated; we thought the sugar curves would be flat-topped. There was at any rate no secondary peak in the P_H curves, as in the titer curves, but it was particularly noteworthy that the P_H curves for glucose and lactose should practically coincide, while the titer curve for glucose was considerably higher than that for lactose, both being, of course, higher than that for the plain mash. We had expected that the secondary peak would be present only in the titer

³ Ibid., 1922, 30, p. 445.

Chart 1.—Acid production of *B. welchii* in plain, glucose and lactose meat mashes.

curve for the plain mash; it occurred, however, also in the sugar medium curves, together with distinct depressions before and after, as in the plain mash medium. These findings raised doubt as to whether 5% of these sugars was really an excess, or, if so, whether the initial peaks of the curves really indicated the exhaustion of the sugar and whether our original interpretation of the secondary peak in the titer curve as due to deaminization was correct.

Turning first to the possibility that *B. welchii* was able to exhaust even 5% of sugar from the medium, a comparison was made of the acid curves for mediums containing in one case as high as 20% added glucose.

The P_H curves were found to be approximately alike for all mediums containing glucose, whether 5, 10 or 20%; a peak was reached and a depression occurred, somewhat tardily it is true, as in the medium without added sugar.

All of the titer curves in this experiment were more flat-topped than usual after the initial peak; those for 5 and 10% glucose were essentially alike and with the control without sugar showed the secondary peaks less plainly than usual. The 20% glucose titer curve showed no secondary peak, but the initial peak was more marked than in the mediums containing less sugar.

Yet the sugar was not exhausted in any of these except the control, as the following data show:

Added Glucose	Final Glucose	Glucose Utilized
%	%	%
0	0	?
5	3	2
10	8	2
20	15.5	3.5

Some attempts to use the usual chemical sugar tests failed, owing to the interference by the nitrogenous constituents of the mediums, but we were successful in removing these by Foster's ⁴ method of precipitation with tannic acid, lead acetate, and sodium oxalate, followed by the Folin-McEllroy ⁵ technic for quantitative estimation of glucose.

This experiment compelled us to discard the idea that the initial peaks of the curves, either of P_H or of titratable acidity, represent the "sugar-free" point and convinced us that even 5% glucose was an excess for this culture under the conditions of the test.

We immediately became interested in determining more exactly how much glucose *B. welchii* could utilize in mediums containing less than 5%. The curves in chart 2 show the result of such an experiment.

⁴ Jour. Bacteriol., 1921, 6, p. 211.

⁵ Jour. Biol. Chem., 1918, 33, p. 513.

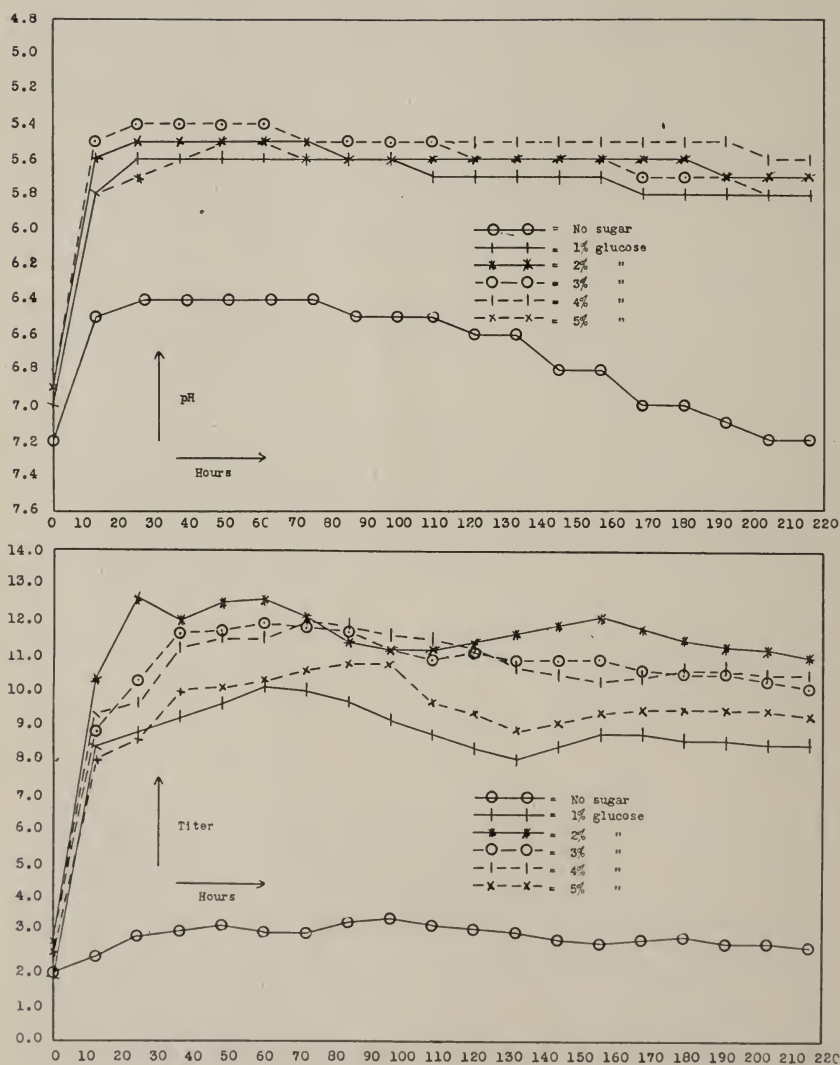


Chart 2.—Acid production of *B. welchii* in meat peptone mashes containing varying concentrations of glucose.

There was nothing significant in the P_H curves; those for the sugar mediums reached a higher acidity than that for the sugar-free mediums, but without marked differences among themselves.

The titer curves were more interesting, with the highest acidity recorded for 2% glucose; then 3, 4, 5, 1 and 0% in the order named.

It is clear that 2% glucose provided optimum conditions for acid production in our tests; more than 2% gave evidence of inhibition and 1% was completely exhausted, as shown by the final quantitative tests whose results follow:

Added Glucose	Final Glucose	Glucose Utilized
%	%	%
0	0	?
1.0	0.0	1.0
2.0	0.4	1.6
3.0	1.5	1.5
4.0	2.5	1.5
5.0	3.4	1.6

These data show that a remarkably definite amount (1.5-1.6%) of glucose was utilized in the various flasks of this experiment, but comparing the results with those of the previous test one might reason that the glucose utilized is within certain limits proportionate to the amount present even though that amount is an excess.

Secondary peaks are prominent in the titer curves for 1, 2 and 5% glucose, less so in those for 3 and 4%. The relatively close correlation of the curves for 1% glucose (less than excess) and for 5% glucose (more than 3 times the amount utilized) again emphasizes the difficulty of employing the titer curve as a criterion of freedom from sugar.

We next endeavored to find an explanation for the recessions in curves representing mediums with excess glucose, in the escape of volatile acids, which Wolf and Telfer⁶ have shown comprise about 50% of the total acidity in a 2% glucose peptone medium.⁶ Tests were made, both with plain meat mash peptone medium and with a similar medium containing 5% glucose, using 2 flasks of each. In each set, one flask was stoppered, as usual, with a cotton plug wrapped in gauze; the other was provided with a sterile rubber stopper which was wired onto the neck of the flask. Through a perforation in this rubber stopper extended a glass tube reaching from near the bottom of the flask and bent over on the outside like a siphon to connect with a short piece of rubber tubing closed with a pinch cock. The purpose of this device was to prevent the escape of volatile acids whose loss we thought might account for the recession in both P_H and titer curves in our

⁶ Bioch. Jour., 1917, 11, p. 197.

previous experiments. The pressure developed within the flasks so stoppered was ample within a few hours to force the culture fluid samples through the siphon when the pinch cock was opened.

The results, however, failed to support our supposition, for both the P_H and titer curves secured for plain meat mash and the 5% glucose meat mash were essentially the same in the rubber stoppered and cotton stoppered flasks.

Assuming from Kendall's work that ammonia cannot be held responsible for a recession in acidity so long as an excess of fermentable sugar remains, and having failed to account for such a recession by the escape of volatile acids, we are now inclined to attribute the loss of acidity that occurs to destruction of certain acids with formation of neutral, or at least less strongly acid compounds, as, for example, carbon dioxide and water from lactic acid. Some of these may recombine to form less dissociable compounds whose presence would be shown in the secondary peak of the titer curves, even while the H^+ ion concentration was falling. This explanation seems now more acceptable than that formerly advanced, in which we assumed the secondary titer curve to be due to an increase in amino acids.

None of our mediums containing excess sugar, in which a recession in the curves occurred, became more alkaline than P_H 6; therefore in utilizing a terminally increased H^+ ion concentration as a qualitative criterion of fermentation, where a neutral medium is used, as recently advocated,⁷ there is no danger of misinterpretation attributable to such recessions.

We then returned to the curious differences in the height of the titer curves for lactose and glucose shown in chart 1. It will be recalled that the P_H curves were essentially alike.

Several ideas occurred to explain the differences in the titer curves. A seemingly remote one was that the primary hydrolysates of lactose, i. e., glucose and galactose, were unequally fermented, and we reasoned that by doubling the amount of lactose (10%) in one flask over that of glucose (5%) in the other, we should obtain identical results. We also compared a medium containing 5% galactose with these.

The P_H curves for glucose, galactose and lactose were practically alike, also the titer curves for glucose and galactose, but the titer curve for lactose (10%) again ranged below those for the monosaccharides.

⁷ *Jour. Infect. Dis.*, 1921, 29, p. 321.

The curves for the disaccharide, saccharose, and the monosaccharide levulose also resemble those for glucose and galactose, although in this experiment the P_H values as well as the titer values for lactose ranged slightly lower than those for the other sugars.

Notwithstanding, the H^+ ion concentration seems to constitute an important limiting factor in the utilization of these sugars by *B. welchii*, and the more marked differences in titratable acidities may some time be explained on the basis of different dissociation constants in the products of lactose fermentation. Possibly more highly ionized products are formed from lactose than from glucose, galactose, levulose or saccharose, and the transformation may be direct or at least without an intermediate hydrolysis of lactose into monosaccharides. We could not, at this time, undertake to determine whether there are differences in the acids formed from these sugars by *B. welchii*, and the literature gives no satisfactory clue.

SUMMARY

The fact that a distinct peak, followed by a depression in H^+ ion concentration and titratable acidity, occurs in peptone meat mash mediums containing an excess of glucose, levulose, galactose, lactose or saccharose undergoing fermentation by *B. welchii*, precludes the possibility of regarding the change in direction of such curves as proof of freedom from sugars.

The escape of volatile acids seems not to be responsible for the recessions in acidity, and the hypothesis is suggested that some of the acid first formed is subsequently destroyed. The resulting products being less dissociable may account for the recession in H^+ ion concentration, and at the same time for the increased titer observed in the secondary peak of the titer curves.

The recessions in acidity, when an excess of fermentable sugar is present, are insufficient to confuse qualitative fermentation tests.

The limiting P_H values for glucose, levulose, galactose, saccharose and lactose are approximately equal; also the titratable acidities for all but lactose; *B. welchii* never produces quite as much acid from lactose as from the other sugars, owing possibly to different dissociation constants in the end products.

NEW MICROMANIPULATOR AND METHODS FOR THE ISOLATION OF A SINGLE BACTERIUM AND THE MANIPULATION OF LIVING CELLS

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Barber's method for the isolation of bacteria by means of mechanically operated pipets has been used with considerable success, not only in the branch for which Barber primarily intended it, but also in experimental cytology and embryology. In 1912, the method was first applied to the dissection and injection of animal cells (Kite and Chambers¹), opening up a new field for investigation into the physical properties of protoplasm and the nature of cell structures.

The moist chamber devised by Barber and his method of making glass pipets and needles (Barber,² Chambers³), which are stiff and yet fine enough to puncture red blood corpuscles, leave nothing to be desired. Unfortunately, his instrument for manipulating the pipets, unless skilfully made, has too much lost motion, and wear and tear soon renders the movement jerky and undependable.

By using a new principle for moving the pipets, I have been able to construct an instrument which has the following advantages over Barber's: (a) simplicity of construction; (b) no lost motion through wear and tear; (c) accurate and continuous control of the movements of the needle or pipet tip in any direction under the highest magnification of the microscope; (d) maintenance of the needle tip in one focal plane while it is being moved back and forth in any of the three directions; and (e) existence of adjusting devices to facilitate placing the needle or pipet into position.

The basic principle of the instrument consists in rigid bars which are screwed apart against springs. The movements imparted are in arcs of a circle having a radius of about $2\frac{1}{2}$ inches. As the extreme range of movement of the fine adjustments is only 2 mm., the curvature of the arc is unnoticeable. The instrument is being patented.

Received for publication, June 9, 1922.

¹ Science, 1912, 36, p. 639.

² Philipp: Jour. Sc., Section B., Trop. Med., 1914, 9, p. 307.

³ Biol. Bull., 1918, 34, p. 121.

A MECHANICAL MICROMANIPULATOR FOR CONTROLLING THE MOVEMENTS OF A MICRONEEDLE OR MICROPIPET IN THE FIELD OF A COMPOUND MICROSCOPE

The principle of this device is demonstrated on considering the mechanism for the movements in one plane only (fig. 1). This consists of 3 bars of rigid metal connected at their ends to form a Z-like figure by resilient metal acting as a spring hinge. On turning certain screws the bars are forced apart; on reversing the screws the bars return to their original position, owing to the springs at the end of the bars. By these means arc movements may be imparted to the tip of a needle when placed in the proper position.

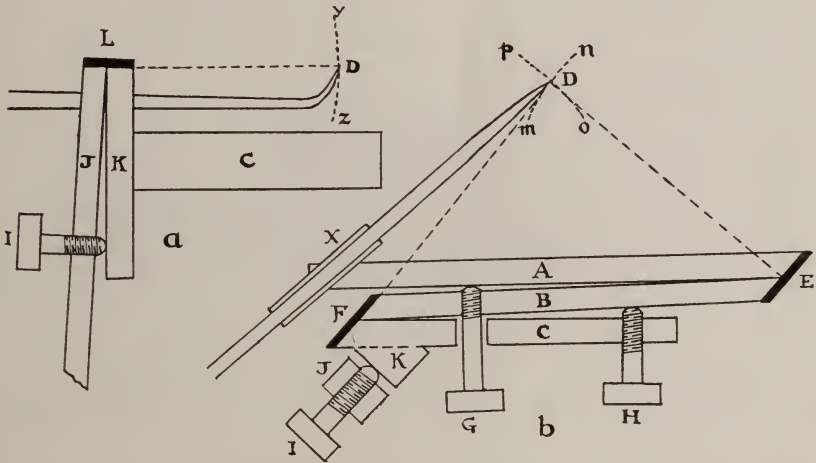


Fig. 1.—Diagram showing the working principle of the micromanipulator. In 1, a, where the instrument is viewed from the side, screw I moves the needle tip through the vertical arc y-z. In 1b, where the instrument is viewed from above, screws G and H move the needle tip through the horizontal arcs m-n and o-p.

The needle or any instrument, the tip of which is to be manipulated, is held in a carrier fastened to the free end of a bar, A, at x. The needle is made to extend so that its tip is at the apex of an imaginary triangle at D. In order to obtain 2 movements at right angles to one another and in the horizontal plane, the tip of the needle must be at the apex, D, of a right-angled isosceles triangle, the base of which is a straight line joining the centers, E and F, of the 2 springs holding the 3 bars, A, B, and C, together. The shank of screw G passes through a large hole in bar C, and is screwthreaded in bar B. Turning it spreads apart bars A and B and imparts an arc movement to the needle tip at D at right angles to that procured by turning screw H.

The movement in the vertical plane at right angles to the aforementioned movements is produced by screw, I (fig. 1, a), which is screw-threaded in a rigid vertical bar, J, and abuts against a vertical extension, K, of bar C. The extension, K, is parallel to the bar, J, and is connected to it at its top by means of a spring hinge. Turning screw, I, spreads apart bars, J and K, and lifts the whole combination (A, B, and C), and imparts an arc movement in the vertical plane to the tip of the needle at D. In order to procure a vertical movement the tip of the needle at D must lie in the same horizontal plane, L-D,

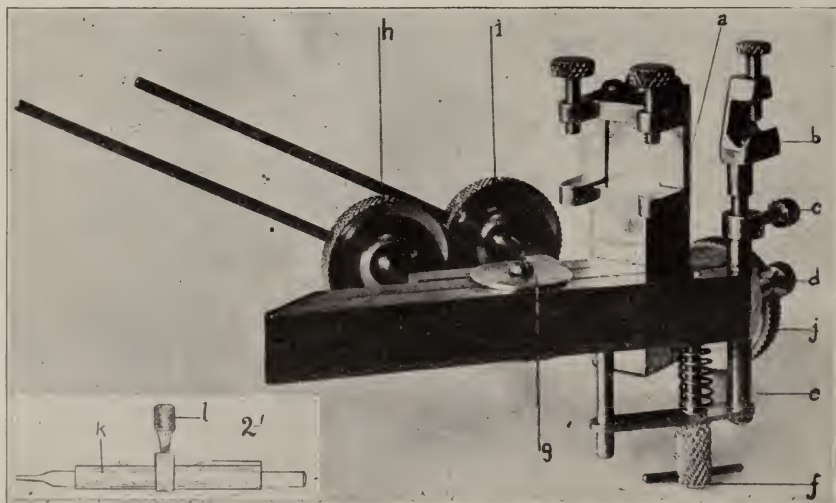


Fig. 2.—Left-handed micromanipulator to be clamped to microscope. a, stationary part with lugs by means of which instrument is clamped to microscope stage; b, holder for brass collar in which pipet is carried; c, screw to clamp post of holder at any desired height; d, screw to clamp main post of holder; e, main post of holder which revolves on its axis to produce a side-to-side coarse adjustment of pipet; f, coarse adjustment screw for raising and lowering; g, upper one of two disc guides for the horizontal bars; h, and i, fine adjustment screws for lateral movements; j, fine adjustment screw for vertical movement.

Fig. 2'.—Brass collar which is to be clamped at k into holder, b, of instrument to serve as coarse adjustment for the in-and-out movement of the pipet; l, screw which presses on a spring to clamp pipet in collar.

with the spring fastening K and J together. When screw, I, is turned, the needle tip will move in an arc, y to z, more nearly vertical than any other arc on the same circumference of which the point, D, is the center.

The micromanipulator may be furnished either with a clamping device (fig. 2, I) for fastening it directly to a square microscope stage (see fig. in article by Kahn⁴), or with a rigid pillar which rises from

⁴ Jour. Infect. Dis., 1922, 31, p. 344.

a large metal base on which the microscope is clamped. When fitted with a clamping device, the instrument depends for its steadiness on that of the microscope.⁵ The horizontal bars of the instrument extend diagonally across the corner below the level of the stage. They do not interfere with the substage accessories of the microscope nor with any of the known types of mechanical stages.⁶

The necessity of having one or two instruments is, of course, conditioned by the type of work to be done. For picking up bacteria one is sufficient. For microdissection in experimental embryology a great deal can be done with one instrument, but for cell injection in general and for tissue cell dissection two instruments are indispensable so that two needles or a needle and a pipet may be manipulated simultaneously.

When two instruments are to be used both must be placed at the front of the microscope so that the needles may extend, side by side, into the moist chamber from the front. As the horizontal bars of the instrument extend diagonally under the microscope stage, one must be a mirror image of the other. According to their position with respect to the microscope, they may be designated as right-handed and left-handed models. For bacteriologic work, in which it is more convenient to work from the left, the right-handed model is to be preferred, as it can be swung around and fastened to the left side so that the pipet may project into the moist chamber from the left.

THE SETTING UP AND THE WORKING OF THE INSTRUMENT

The instrument possesses devices to aid in the preliminary adjustment of the pipet or needle. One is the brass collar, fig. 2'. The pipet is first inserted into the collar where it is held in place by a spring. The collar is then clamped in the pipet holder of the instrument. This device facilitates sliding the pipet into or out of the moist chamber without danger of breaking or contaminating the tip of the pipet. For raising and lowering there are two adjusting devices. One is the telescoping pillar (fig. 2, c), for quickly adjusting the pipet to the height of the chamber; the second is operated by a spring screw (fig. 2, f) for bringing the pipet into focus. By these means the pipet tip is brought into the field of a low powered objective. Before centering the tip one must set the bars which control the fine adjustments into a state of tension by giving a few turns to the milled heads of each of

⁵ Steadiness may be assured by a brace, one end being screwed to the rigid vertical part of the instrument and the other end to the foot of the microscope.

⁶ In the case of the Bausch & Lomb and Spencer stages it may be necessary to replace the screw clamping the front end of the stage for one with a shorter head.

the three screws. The needle tip is then more or less accurately centered and finally raised close to the hanging drop by means of the preliminary adjustments. The instrument is now ready for action.

The milled heads of the screws which control the lateral movements (fig. 2, h and i) may be provided with levers to increase the delicacy of their manipulation. The screw, j, controlling the vertical movement may be furnished with a wire wound flexible shaft about 18 inches long. Curving the shaft around one side of the microscope brings the control of this screw, which is the one most frequently used, close to that of the fine adjustment of the microscope. The shaft also facilitates the use of both hands for the various movements of the one instrument (see fig. in Kahn's article).

The micromanipulator is intended to be used with the mechanical stage of the microscope. The mechanical stage moves the moist chamber. As the cell or tissue to be manipulated lies in a drop hanging from the roof of the chamber, the motion imparted by the mechanical stage moves the cells against the microneedle. Indeed, most of the dissection, when a single needle is used, is done by first bringing the needle tip into the cell and then dragging the cell away by means of the mechanical stage.

The horizontal movements of the micromanipulator are used mostly for the purpose of bringing the tip of the needle accurately into a desired spot in the field of the microscope preparatory to the actual operative work. In order to insure the greatest possible steadiness to the vertical movement, the part of the instrument which produces this movement adjoins, and is manipulated from, the stationary and rigid part of the instrument. To make this possible the present design incorporates a theoretical error which can be understood from fig. 1. Turning screw I to produce the vertical movement throws the combination of bars A, B, and C out of the horizontal, and it is these bars on which the lateral movements of the needle depend. However, the angle at which these bars are placed minimizes the error so as to be practically unnoticeable.

Guides exist in the instrument to insure a true travel of the bars as they spread apart or come together. The guide for the bar which produces the vertical movement consists of a depression in the stationary part of the instrument into which the vertical bar fits. The guides of the lateral movements are two metal disks which can be tightened or loosened by screws. The upper one is seen in fig. 2, f.

They correct two possible errors which may occur on reversing the direction of movement, namely, a dropping of the needle or pipet out of focus and a shifting to one side.

The first error can be corrected by tightening one or both of the guides; the second, by loosening them. The guides, therefore, must be neither too tight nor too loose. The first error is the more serious of the two. It is due to an unequal tension in the springs which throws the tip of the moving screw to a different spot on the bar against which it abuts. If this be not corrected, the screw will, in time, wear a depression in the brass bar that is out of center, thus perpetuating the error. The second error is due to the fact that the guides are too tight so that they bind and prevent the bars from making a true return. If not corrected, this error will gradually be eliminated with the wear of the frictional surfaces.

By an accidental knock the horizontal bars of the instrument may be jarred out of place or the fine adjustment screws injured. If the upper and lower surfaces of the horizontal bars are not flush, loosen the guide disks (fig. 2, f) also the screws of the springs on the ends of the bars and, with a wooden mallet, gently hammer the bars until they are flush. Then tighten the guide disks to keep the bars flush and carefully tighten the screws of the springs. If the screws have been bent by the accident they must be changed, otherwise tightening them will again pull the bars out of place. If the guide disks are bent, they also must be changed. A more serious accident occurs when the fine adjustment screws are injured. The steel shafts of the screws may be bent or they may have cut into the brass so as to loosen the threads. This tends to throw the shaft of the screw out of center. In such a case a new screw must be procured and accurately centered opposite the bar against which it abuts.

THE SUBSTAGE CONDENSOR AND THE METHOD OF MAKING BARBER'S MOIST CHAMBER AND GLASS NEEDLES

For critical illumination the height of the moist chamber must be equal to the working focal distance of the substage condensor. The Abbe condensor can be used by removing the top lens. The focal distance of the remaining lens is almost one inch. In the Bausch and Lomb microscope the substage can easily be arranged to raise this lens sufficiently to have at least half its focal distance above the surface of the stage. This is ample if one is satisfied with a moist chamber no

higher than half an inch. The focal distance of this lens can be reduced and its illuminating power correspondingly increased by placing the lens of a 10 X dissecting lens on top of it. This combination has a focal distance of about $\frac{3}{8}$ of an inch and, if the substage can be raised to bring the top lens flush with the upper surface of the stage, all of this distance may be used for the height of the moist chamber. Better results are secured with a triple lens condensor with its top lens removed. Such a condensor from Leitz, which I am using, has a working focal distance of just $\frac{3}{8}$ of an inch. One may also use condensers which are made with a specially long working distance for projection apparatus in which a cooling trough is placed between the condensor and the slide.

If the working focal distance of the condensor is less than $\frac{3}{8}$ of an inch, it is well to have two moist chambers, one for critical work and the other, from $\frac{3}{8}$ to $\frac{1}{2}$ inch high, for ordinary work. This is advisable because it is easier to make needles for the higher chamber.

The moist chamber is made of glass. There is convenient form for cytological purposes with the open end designed to face the front of the microscope. The base is a fairly thin glass slide about $2\frac{3}{4} \times 2$ inches in size. The sides consist of strips of plate glass about $1\frac{7}{8}$ inches long and $\frac{1}{4}$ inch wide and of a height determined by the available condensor. One end of the chamber is closed with a strip of glass of the same height as the sides and backed by another strip a fraction higher in order to prevent a coverslip from sliding beyond it. The trough of the chamber should be from $\frac{3}{4}$ to $\frac{7}{8}$ of an inch wide. The strips are cemented with any ordinary glass cement. Heated Canada balsam serves well. Care must be taken to have the upper surfaces of the strips horizontal. This may be done while the cement is still soft by focusing on the upper surface of the strips and by manipulating the strips until all parts of their surfaces lie in one focal plane.

To maintain moisture in the chamber strips of wet blotting paper may be placed across its inner end and along its sides.

This moist chamber is designed for coverslips 24×40 mm. The coverslip is sealed on the chamber with petrolatum. Square or round coverslips may also be used provided the rest of the chamber be roofed with other strips of coverglass.

The hanging drop containing the cells or tissue to be operated on is placed on the coverslip, which is then inverted over the moist chamber.

To prevent the petrolatum from spreading on the coverglass and from contaminating the hanging drop a thin film of melted paraffin may be spread and cooled on the coverglass bounding the area to be occupied by the hanging drop.

The moist chamber is open at one end to permit the entrance of the microneedles or pipets. To prevent undue evaporation, especially when a preparation is to be left over night, the open end may be temporarily closed by means of a paraffined, thin cardboard trough. The trough is placed over the shanks of the needles and filled with soft petrolatum containing a few threads of cotton to give substance to the petrolatum. The petrolatum closes around shafts of the needle and seals the opening of the chamber without interfering with the movement of the needles. To prevent the petrolatum from spreading on the floor of the moist chamber it is well to have a shallow pan of cardboard set under the shanks of the needles for the trough to rest on.

The needles are made either from soft or hard glass tubing. When a brass collar is used (fig. 2) the glass tubing should be selected to fit it. The collars furnished with the instrument receive tubing about $\frac{1}{8}$ inch in outside diameter. As regards the wall of the glass tubing in general, it seems to be true that the thicker the wall the firmer tends to be the tip of the needle. The method of making the needle is given in a paper of Barber's² and in one of mine.³ A brief account will suffice here. Acetylene or ordinary illuminating gas may be used. For a microburner use a piece of hard glass tubing bent at right angles and with the burner end closed except for the smallest aperture that will retain a flame. This may be done by heating the end and pinching it with forceps. The size of the flame may be regulated by a screw pinch cock on the rubber tube.

To make the needles proceed as follows: 1. In an ordinary burner draw out one end of a glass tube with a capillary of about 0.3-0.5 mm. in diameter. 2. Lower the flame of the microburner to the smallest flame possible. Hold the shank of the tube in the left hand and grasp the capillary at its end either with the thumb and finger of the right hand or with forceps having flat tips coated with Canada balsam. Bring the capillary over the flame and pull gently until the capillary parts. The hands should remain on the table during the process and, as the capillary parts, lift the glass away from the flame by turning the hands slightly outward. The capillary will separate with a slight tug. The tip should be like that in c. If too little heat is used

and the pull made too suddenly, the capillary will part with a snap and have a broken tip. If too much heat is used the tip will be drawn out into a long hair, e. 3. Bend the capillary at right angles by heating it just back of the point and pushing up with a dissecting needle, b. The length of the needle beyond the bend is conditioned by the height of the moist chamber to be used. The type of needle shown in g is used for cutting by bringing the upper limb of the needle below and up into the cell. 4. The micropipets are made from needles of the kind shown in d. The needle is first inserted in the brass collar (fig. 2') and connected with a rubber tube to be blown into by mouth or with my micro-injection apparatus (cf. Chambers⁷). It is then placed in the carrier of the instrument and the tip brought up into a droplet suspended in the moist chamber. By jamming the tip against the cover-glass the hair tip breaks off converting the needle into a pipet. During this procedure blow into the hollow needle to prevent clogging of the pipet by glass fragments which tend to be drawn in by capillarity.

OTHER APPARATUS HITHERTO USED FOR MICRO-OPERATIVE WORK

Barber's instrument is based on the principle of a carrier pushed along a groove by a screw at one end. By having a series of three carriers built up on one another, each traveling in a different direction, movements in any one of three dimensions may be imparted to a needle clamped to the top carrier. Hecker⁸ improved Barber's instrument, but added materially to the intricacy of its make-up.

Other investigators that I know of who have devised instruments for micro-operative work are Schmidt,⁹ Chabry,¹⁰ Schouten,¹¹ Tchahotine,¹² McClendon,¹³ Malone,¹⁴ Bishop and Tharaldsen.¹⁵

Schmidt's instrument is one of historic interest only. I have already described it.³ Chabry used a delicate spring device with which he could shoot the tip of a glass needle into an ovum to any desired depth. Schouten uses his for the isolation of bacteria. It consists of a pillar carrying a needle which may be mechanically raised or lowered. For

⁷ Anat. Rec., 1922, 24 p. 1.

⁸ Jour. Infect. Dis., 1916, 19, p. 306.

⁹ New Orleans Med. Jour., 1869, 22, p. 627; 1870, 23, pp. 66 and 274.

¹⁰ Jour. de l'Inst. et de Physiol., 1887, 25, p. 167.

¹¹ Ztschr. wiss. Mikrosk., 1905, 22, p. 10; Königl. Akad. Wetensch. Amsterd., 1911, 13, p. 840.

¹² Ztschr. wiss. Mikrosk., 1912, 29, p. 188; Biol. Centralbl., 1921, 32, p. 623.

¹³ Biol. Bull., 1907, 12, p. 141.

¹⁴ Jour. Path. & Bacteriol., 1918, 22, p. 222.

¹⁵ Amer. Nat., 1921, 55, p. 381.

the horizontal movements, Schouten depends on pushing the microscope on a flat base. McClendon attached an up and down movement to a Spencer mechanical stage. Tchahotine used a mechanism attached to the tube of his microscope from which extended a glass needle curved in such a way as to bring its tip into the microscopic field where it was brought into focus. Tissues are dissected under a low power objective by moving the microscope tube and by pushing the cells against the needle tip by means of the mechanical stage of the microscope. Malone uses Schouten's method, but, instead of having a special pillar with a raising device, he mounts his pipet carrier on the tube of a second microscope whose adjustments serve as a means for raising and lowering the pipet. Bishop and Tharaldsen have a simple instrument based on a principle somewhat resembling mine but lacking in proper control for one of the two lateral movements. Recently I have heard that Zeiss is manufacturing a microdissection instrument which, however, is said to be of an intricate design.

CHAMBERS' MICROMANIPULATOR FOR THE ISOLATION OF A SINGLE BACTERIUM

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As I have been working for several years on the isolation of bacteria with Barber's apparatus and have recently been using Chambers' instrument for the same purpose, Dr. Chambers suggested that I make some comments on the relative merits of the two instruments together with a short review of Barber's isolation method.

The mechanical principles involved in the construction of this instrument are entirely different from those of Barber's pipet holder, while the basic methods of manipulation are essentially the same. There are several noteworthy features in the Chambers' instrument which modify and improve the original Barber technic. These features may be best described under two headings: (1) advantages in construction, and (2) advantages from the use of various accessories to aid in manipulation.

ADVANTAGES IN CONSTRUCTION

The mechanical principles on which the instrument is based are fully described in the preceding article by Chambers.¹ The absence of parts which may loosen by wear and tear renders possible great precision in the manipulations. While excellent work may be done with the Barber pipet holder, the parts wear somewhat after several months' use, giving rise to a certain amount of false motion. For instance, when one desires to move the pipet laterally one may encounter an unexpected vertical motion. The Chambers apparatus used by me had been in use for two years, and in spite of this I was unable to detect any false motion.

A second advantage is that the instrument clamps directly on the stage of the microscope giving much greater rigidity than is possible with the metal flange which has to be attached to the stage of the microscope when Barber's instrument is used. Third, the smaller size of the instrument brings all manipulations closer to the microscope and

Received for publication, June 9, 1922.

¹ Jour. Infect. Dis., 1922, 31, p. 334.

eliminates accidental jostling of the pipet holder which may shift the needle out of focus. This compactness also makes it feasible to use a short pipet which is easier to focus and is in less danger of contamination as less of the pipet is exposed.

ADVANTAGES OF THE ACCESSORIES IN CHAMBERS' APPARATUS

Barber's instrument possesses no accessories so that all the adjustments, both preliminary and operative, have to be made by means of the same finely threaded screws with a consequent waste of considerable time.

In Chambers' instrument the several accessories are as follows:

1. There is a brass collar (fig. 2') through which the shank of the pipet is inserted before clamping it in the pipet carrier of the instrument. Besides insuring rigidity to the pipet and thus greater accuracy for manipulation, the collar facilitates bringing the pipet into the field of the microscope. It also steadies the pipet as it is being withdrawn from the moist chamber, thus minimizing contamination or injury to the delicate tip.

2. For the vertical manipulation of the pipet there are three different adjusting devices: first, the telescoping pillar for roughly adjusting the pipet to the height of the moist chamber, after which it may be tightly clamped; second, another coarse adjustment operated by a spring screw with which one may bring the pipet into focus, and, third, the fine adjustment of the knurl headed screw (fig. 1), which is used in the actual operation of isolation. The first two devices are for the coarse adjustment and enable one to use moist chambers of practically any height. They aid greatly in the technic of the vertical adjustment, which is the most important one from the bacteriologic point of view.

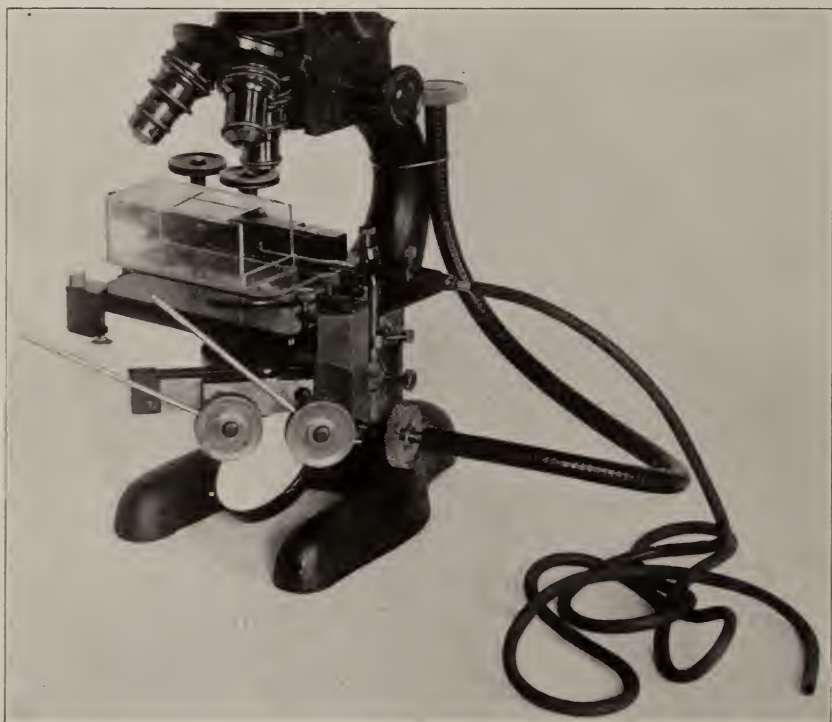
3. The use of levers on the screws controlling the lateral movements insures great delicacy to the touch and is a decided aid in bringing the pipet directly under the droplet containing the organism, especially when working with the higher power or oil immersion lens where the slightest movement is greatly magnified.

4. The flexible shaft attached to the vertical control screw brings the movement of that screw behind the microscope away from all working parts and close to the fine adjustment of the microscope.

For bacteriologic work it is more convenient to have the moist chamber as Barber originally devised it, viz., with its open end so placed that the pipet may project into it from the left side. This

facilitates the frequent interchange of pipets so necessary in isolating bacteria. For this purpose it is necessary to have the pipet holder attached to the left side of the microscope. This can be done with the form which Chambers designates as right-handed by fastening it on the left side of the microscope stage near the outer corner (fig. 1).

In the preceding article it has been recommended to have the height of the moist chamber equal to the working focal distance of the sub-



Micromanipulator mounted on the left side of the microscope for isolating bacteria. Note Barber's moist chamber with the coverslip marked with cross lines to aid in locating areas. The chamber shown here is higher than necessary.

stage condensor. This limits the height to one which may make the chamber too shallow to be convenient for frequent interchanges of the pipet. I, therefore, use a chamber $\frac{3}{4}$ of an inch high, 1 inch wide and $2\frac{1}{2}$ inches long. It will be noted that this moist chamber is not only deeper but also wider than the one Chambers uses for cytological work.

THE ISOLATION METHOD

For the isolation of a single bacterium one must have the under surface of the coverslip so treated as to hold minute droplets without fear of their spreading or possibly running together. The droplets placed on the coverslip must be slightly hemispheroidal in order that their outlines may be distinct and all parts of it clearly visible. Also, in order to maintain these droplets throughout the operation, the moisture conditions within the chamber must be sufficient to prevent their evaporation and at the same time must not be too great for fear of flooding them.

It is necessary, therefore, to have the surface of the coverslip specially prepared. Barber, after smearing the cleaned coverslips with petrolatum, washes them with soap and water to get rid of the excess of petrolatum. The coverslips are then carefully cleaned with a dry cloth, heated enough to soften the petrolatum and rubbed again while still warm. The aim is to remove as much petrolatum as possible without the use of excessive heat or any fat dissolving reagent other than soap. If an excess of petrolatum is left on the cover, small particles will appear in the droplets and may be mistaken for bacteria. If all petrolatum is removed, the droplets run together and make successful isolation impossible. Instead of soap and water one may use 95% alcohol with equally good results. One must realize that success or failure in isolation work depends on a proper treatment of the coverglass.

The method of procedure for the isolation of a bacterium may be summarized as follows:²

1. Prepare a young liquid culture from a subculture not more than 18 hours old.
2. Insert the tip of a needle into a tube of the liquid culture and convert the needle into a pipet by gently rubbing it against the wall of the tube. Then with a rubber tube on its shank suck up a small amount of the culture.
3. Insert the pipet in the brass collar, then clamp it in the pipet holder of the instrument and bring the tip into focus in the center of the microscopic field (see figure). Raise the pipet until its tip touches the undersurface of the coverslip and expel an appreciable droplet. This may have to be diluted with sterile fluid if the culture is too dense.
4. After securing a moderately dilute preparation fill the same or a new pipet to a little below its bend. Lower the pipet, and with the aid of the mechanical stage, bring another portion of the coverslip into view. By alternately raising and lowering the pipet a series of minute droplets will be produced on the coverslip wherever the pipet touches it. The fluid runs out by capillary attraction and needs no blowing. Some of these droplets will be found to contain a single micro-organism.

² The method of making the pipets is described in the preceding article by Chambers.

5. Replace this pipet with a new sterile one containing a small amount of sterile liquid medium which must not run below the elbow. This new pipet is now brought directly under a droplet containing a single micro-organism. The pipet is then slowly raised and as soon as it touches the surface the droplet with the contained organism will flow into it. This occurs by capillary attraction and no suction is required.

6. This pipet, which is known to contain only one micro-organism, is carefully removed from the apparatus and its tip inserted into a tube containing a suitable sterile medium. The entire contents of the pipet are now to be expelled by blowing. As an added precaution it is well to break off the tip of the pipet in the culture medium. The blowing may be done by mouth or by a rubber bulb operated either by the hand or the foot.

THE RELATION BETWEEN THE FIXED AND FREE SALTS OF BACTERIA *

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In their study on the conductivity of bacterial cells, Green, Minneapolis and Larson ¹ have shown that, at death, there is a rapid exosmosis of salts from the bacterial bodies when suspended in water. Their work suggests that some of the salts at least are free within the cell of the organism.

The present study was inaugurated for the purpose of determining the nature and relation between the fixed and free salts of bacteria, and in particular with *B. coli*. In this paper the salts which diffuse out of the killed bacteria are referred to as the free salts, while those which do not diffuse out but are found in the ash of bacteria which have been dialyzed, are called the fixed salts.

The bacterial cell, although minute, may nevertheless be a complex and well organized system. We cannot determine any particular specific structures in the cell, but we know that it is composed of solid particles of protoplasm suspended in a liquid medium.

This liquid, besides keeping the cell turgid against the pressure of the surrounding medium, serves as a carrier for the particles of food from the inside of the cell wall to the cell substance, and also as a carrier for the waste products of the cell back to the cell wall to pass from there into the surrounding medium. No regular circulatory system has ever been demonstrated in bacteria, but there must be some means of carrying the ions of salts, as well as other particles in solution, which have passed through the cell wall, to those parts of the cell where they are built up into new protoplasm. Whether or not the liquid passes through the cell in a regular path, carrying with it particles of food or carrying away waste materials, or whether the substances in solution simply move about in the liquid as a dispersion medium, from which they are adsorbed to the cell structures, has never been determined.

Received for publication, June 10, 1922.

* Supported by a grant from the National Dental Research Association.

¹ Jour. Infect. Dis., 1922, 30, p. 550.

It has frequently been pointed out that the membrane of bacteria may be lipoidal in nature. Lipoids, being surface tension depressants, would have a tendency to gather in the surface layer. Whatever this membrane is, it seems to function as an osmotic one. Particles pass into the cell wall, due to the pressure of the solution on the outside, and are released from the inner cell wall by the reduced pressure of the liquid inside of the cell. Whether the cell membrane shows any selective action to particles passing through it, or whether the whole process is simply a physical one of adsorption and osmosis, regardless of the specific nature of the particles, is still a matter of doubt. Some evidence for the former theory has been presented by Cramer² when he pointed out that organisms show a selective action toward phosphate as against chlorid. Again, the fact that a certain amount of chlorid is taken up by the cell and apparently, as will be pointed out later, is not utilized in building up the cell substance, is evidence of little or no selective action on the part of the cell membrane. Chlorid may, however, have a purely physiologic function, which is not yet clearly understood.

There are, then, two distinct groups of salts present in the living bacterial cell. The structural salts are those which are tied up chemically or form a part of the protoplasm of the cell. It would seem, if there is any definite or constant composition of this cell protoplasm, that the nature and amount of the structural salts should remain relatively constant, at least for the same organism, regardless of the conditions of growth. The unbound salts, however, performing, as we think of them, purely physical functions as equalizing the osmotic pressure, and preserving the turgor of the cell, must vary in amount and to a certain extent in composition with the concentration and composition of salts in the medium. An increase of the total concentration of salts in the medium would bring about an increase of salts in the cell liquid. An increase of either sodium, potassium, chloride, or phosphate in the medium would cause an increase of the corresponding substance in the cell contents. The osmotic pressure, for example, of sodium on one side of the membrane would be equalized by an equal pressure of sodium on the other side. Whether this equality of pressure for each element is maintained is not known. We know from data of former investigators² that cells do pile up concentrations of certain elements above those of the same element in the medium. Especially is this the

² Arch. f. Hyg., 1897, 28, p. 1.

case when a particular element is essential to the life process or structure of the organism and is present in comparatively low concentration in the medium. All that this may mean, however, is that the cell structure requires relatively constant amounts of certain essential elements and extracts these from the medium, no matter how low the concentration may be. Such elements then being bound up in the cell structure have little or no effect on the osmotic pressure. The excess concentration of certain salts in the cell over that in the medium may be entirely in the fixed salts, and therefore may not conflict with our theory of osmotic pressure balance.

A plausible conclusion to draw from all this is, first, that the fixed salts remain constant under different conditions of growth, at least with the same organism, and second, that the wide variations found by Cramer and others in the ash content of an organism grown on mediums of different salt concentrations are variations in the free salts only.

All of this work on diffusible salts in cells is comparatively new, and as yet there are no available data to prove or disprove the statement just made. It remains, therefore, for the present a plausible conclusion and nothing more.

In most animals and plants certain cells or groups of cells have purely structural, others purely physiologic, functions. In bacteria in a very general way certain elements compose the cell structure, while others take part in metabolism. The salts combined in the former are the structural, those connected with the latter the physiologic salts.

Another question still to be answered about the diffusible salts is the one concerning the manner in which these salts are held by the cell during life. They may be adsorbed to the cell wall prior to passing into or out of the cells; they may be adsorbed to the cell substance within before actual chemical combination; or they may be in solution in the cell liquid. The broadest assumption and the most logical one would be that they occupy not one, but all three of these places, and fulfil all three functions.

Previous investigators, giving no heed to the fact that cells give off salts when killed, may have lost at least a part of these loosely held salts in the preparation of their samples for analysis through a series of washings and extractions. DeSchweinitz and Dorset³ state as much in their paper on the ash analysis of the tubercle bacilli. It is significant for our purpose that these two investigators found no chloride in their

³ Centralbl. f. Bakteriol., I, O., 1902, 33, p. 993.

samples, which in the present analysis does not appear in the ash but in the diffusate. It is easily seen that a part of the salts, and with that the chlorides, might have been lost by numerous washings with hot water. Also the sulphate, absent in the analyses of DeSchweinitz and Dorset, was found in this work mainly in the free salts.

The part of the mineral matter of bacteria which we here call the fixed salts may be held in the cell in several ways. They may be contained in the cell as simple insoluble inorganic salts or they may be tied to the protein molecule by adsorption or in the form of protein salts. The latter may be true especially of the heavy metal ions.

Previous to this work a rapid qualitative analysis had been made of the ash of bacterium coli. Ca, Mg, Na, K, Fe, Cl, SO_4 and P_2O_5 were found present. The method of procedure in carrying out the work of this paper is as follows: The organisms, a strain of bacterium coli, were grown on ordinary meat extract peptone broth containing 0.5% NaCl. After inoculation the flasks were incubated for 48 hours at 37 C. The organisms were then separated from the medium by centrifuging, and weighed. They were washed in distilled water to remove the broth still adhering to them and again centrifuged from the wash water. After suspending the cells again in about 6 liters of distilled water, they were killed by heat to 60 C. for 30 minutes. Immediately after heating the suspension was cooled, diluted with distilled water and allowed to stand for several hours in order to allow the salts to diffuse out. Again the liquid was separated from the organisms by centrifuging. The diffusate was evaporated to dryness and ignited to burn off the small amount of organic matter present. The organisms left after diffusion were dried and weighed and then reduced to ash in platinum. Both the ash and diffusate were analyzed quantitatively. Cl, Fe, Ca, P_2O_5 , Mg, SO_4 , Na and K were determined.

Green and Larson, in their conductivity experiments on bacterial suspensions, found that the electrical conductivity of the suspension remained constant for about 2 hours. After that the resistance suddenly dropped, showing that the salts do not begin to come out of the live cells until they have been suspended in distilled water for about 2 hours.

DATA FROM ANALYSIS

Total weight of moist organisms.....	114	gm.
Weight of dry matter in organisms.....	12.9260	gm.
Ash + trace of carbon.....	0.8392	gm.
Ash	0.7050	gm.
Amounts of different elements present in the ash		
Cl	0.0000	gm.

Ca ₃ (PO ₄) ₂	0.2440 gm.
Ca	0.0936 gm.
PO ₄	0.1504 gm.
Mg ₂ P ₂ O ₇	0.1935 gm.
Mg	0.0417 gm.
Ba SO ₄	0.0302 gm.
SO ₄	0.0125 gm.
Fe ₂ O ₃	0.0236 gm.
NaCl + K Cl	0.2157 gm.
K Cl O ₄	0.3175 gm.
K	0.0913 gm.
Na	0.0184 gm.
Mg ₂ P ₂ O ₇	0.2642 gm.
P O ₄	0.2251 gm.
Weight of difusate dried	3.5318 gm.
Free salts + carbon	0.9425 gm.

Amounts of different elements in the free salts

AG Cl	0.2857 gm.
Cl	0.0700 gm.
Ca ₃ (PO ₄) ₂	0.2223 gm.
Ca	0.0864 gm.
PO ₄	0.1359 gm.
Mg ₂ P ₂ O ₇	0.0882 gm.
Mg	0.0192 gm.
Ba SO ₄	0.1162 gm.
SO ₄	0.0410 gm.
Fe ₂ O ₃	Trace
NaCl + K Cl	0.6470 gm.
K Cl O ₄	0.3375 gm.
K	0.0936 gm.
Na	0.1863 gm.
Mg ₂ P ₂ O ₇	0.2944 gm.
PO ₄	0.2520 gm.
Carbon	0.01 gm.

DATA IN PER CENT.

Total dry matter in organisms	11.34%
Total ash in the dry matter	12.75%
Fixed salts in the ash	42.79%
Free salts in the ash	57.21%

Percentage of elements calculated on the dry weight of the ash.

Cl	0.00%
Ca ₃ (PO ₄) ₂	35.61%
(Ca O 13.77%, P ₂ O ₅ 21.84%)	
MgO	5.92%
SO ₄	1.78%
Fe ₂ O ₃	3.35%
K	12.95%
Na	2.61%
P ₂ O ₅	33.99%
Total	96.21%

Percentage of elements calculated on the dry weight of the free salts.

Cl	7.40%
Ca ₃ (PO ₄) ₂	23.59%
(CaO 9.13%, P ₂ O ₅ 14.46%)	
Mg O	2.04%
S O ₄	4.36%
Fe ₂ O ₃	Trace
K	9.94%
Na	19.77%
P ₂ O ₅	26.84%
Carbon	1 +
Total	96.94%

An analysis was made also of the mineral constituents of the broth used for the cultivation of the organisms. The results of this analysis are as follows:

Percentage of ash in the broth	0.9%
Percentage of constituents of the ash in broth	

NaCl	0.5%
P ₂ O ₅	0.2%
Ca + Mg	0.1%
Small amounts and traces of K, SO ₄ and Fe.	

In looking over the results of this work, we find that the contents of dry matter and total ash for bacterium coli come within the range found by other investigators for other organisms. The water content seems to be relatively constant for all organisms analyzed up to the present time.

A significant factor for our purpose is that the weight of the free salts is greater than that of the fixed salts. This shows that not only do salts come out of bacterial cells when the latter are killed by heat and suspended in distilled water, but the greater part, 57.21% of the total salts in the cells, diffuse out on the death of the organisms. The total absence of chloride in the fixed salts and its presence in the unbound salts shows, according to our theory, that chloride is not essential in the cell structure but is used to equalize the pressure of chloride in the medium or in some other unknown function. Sulphates are present to a much greater extent in the diffusate than in the ash. Iron is present almost entirely in the fixed salts. The function of iron in the cell is not known. If it serves, as in the cells of higher animals, as an oxygen carrier, its function is a biologic one. Phosphate is by far the most abundant element. It seems to be the essential element in the cell structure, as well as in other functions of the cell, and is often found in higher concentrations in the organisms than in the medium.

Sodium and potassium show interesting results. Both are found in the free salts, but sodium predominates. This was to be expected. With a concentration of 0.5% NaCl in the broth the organisms would have to take up a considerable quantity of sodium to equalize the osmotic pressure caused by the sodium ions in the broth. The fact that this element appears almost entirely in the diffusible salts suggests that its action in the cell is probably physiologic or physical. The results for sodium and potassium obtained here agree very well with analyses of cells of higher plants and animals, in which potassium but no sodium is found present. Potassium is invariably the constituent of cell substance while sodium is present in the fluids that have purely physiologic functions in the bodies of animals and in plants. In red blood cells, for instance, potassium is always present, but sodium has never been found. The large amount of sodium found by Cramer and others in bacteria must be almost entirely in the unbound salts. It is not improbable that the small amount of sodium in the fixed salts could be replaced by potassium by a little higher concentration of that element in the medium. The amount of potassium present in the

diffusate is almost four times as large as the amount of sodium in the ash. This shows that potassium is utilized by the cell in its structure and also has a biologic function.

SUMMARY

It has been shown again that salts diffuse out of bacterial cells when they are killed by heat and suspended in distilled water. Moreover, it has been shown that the amount of free salts in bacteria is greater than the amount of fixed salts. The two groups of salts contain about the same constituents in somewhat different proportions. Chloride and iron are the exceptions, occurring only in the diffusate and in the ash, respectively. The results for sodium and potassium agree well with those found, in the past, in analyses for the same elements in higher plant and animal cells.

The results obtained in this investigation may be typical of other organisms cultivated under similar or different conditions. This fact would, however, have to be determined by added experiments. The results can be regarded as established only for *B. coli* cultivated and treated as described in this paper.

THE ACTION OF THE VISIBLE SPECTRUM ON COMPLEMENT

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The inhibitory power of the invisible (ultraviolet) rays on complement has received extensive recognition. Recent studies by Baroni and Jonesco Mihailesti,¹ Abelin and Stiner,² Courmont, Nogier and Dufourt,³ Bovie⁴ and Brooks⁵ have definitely established this particular phase of photodynamic destruction of complement and amboceptor. Lundberg⁶ recently found that the speed of photo-attenuation of complement markedly increases as attenuation proceeds. McCoy, Hill and Schmidt,⁷ on the other hand, showed the protective action of the aromatic amino-acids (tyrosin and phenylalanin) for complement and amboceptor against the action of these rays. The bulk of this work has been done by direct exposure of serums to the ultraviolet rays varying the thickness of the exposed liquids, or by dilution ending with a simple complement titration. It would appear, then, that attempts to study the effect of the visible rays on complement and amboceptor are entirely lacking. In the present work the action of the visible part of the spectrum (rays of greater wave lengths) was studied to determine their effect on complement.

Methods of Study.—The source of light employed was a powerful 15 ampères tungsten filament lamp of 944 candles per square centimeter operating on 17 volts. The beam of light of this lamp was thrown through a set of projecting lenses and focused on the slit of a large Hilger's spectrograph containing a glass prism. The width of the slit was 1 mm. and its length 7.5 mm. The serums of guinea-pigs were exposed in the spectrum in a glass spectrum cell carrying exactly 1.5 c c of fluid and having a diameter of 4 mm. Care was taken not to fill the cell to capacity surpassing the upper or lower margins of the

Received for publication, June 12, 1922.

¹ Compt. rend. Soc. de biol., 1910, 68, p. 393.

² Ztschr. f. Immunitätsf. u. Exper. Therap., 1913, 19, p. 1.

³ Compt. rend. Soc. de biol., 1913, 74, p. 1152.

⁴ Jour. Med. Res., 1918-19, 38, p. 355.

⁵ Ibid., p. 345.

⁶ Compt. rend. Soc. de biol., 1921, 85, p. 758.

⁷ Jour. Infect. Dis., 1919, 25, p. 335.

spectrum. At the side of the exposed serum the control half of the serum was kept in a small test tube wrapped in black paper. The average temperature of the laboratory was 25 C. and throughout the experiment the room was kept dark. The exposures were continuous and for varying periods of time. Serums (0.2 c c) were dried in air on glass slides and then exposed in a dried state. Dilutions of fresh serums were made up to 10% and exposed. In addition to this set of experiments, a trial was made to use the direct rays of the sun by means of a Fuss heliostat, focusing the image of the sun on the slit of the Hilger's spectrograph. So far exposures to the solar spectrum have been of short duration, 2.5 and 4.5 hours, respectively. As regards the loss of light, it may be said that altogether about 20% of the total light passes through the prism so that a considerable amount of light is lost by the use of the lenses and spectrograph. The spectrum was further divided into 2 and 3 regions and complements exposed to the rays of each region. The 2 regional divisions were: (a) reds, orange and yellow, and (b) green, blue and violet. The solar spectrum was divided in 3 regions because of the greater lengths of the red and infra red rays. These regions were: (a) infra reds and reds; (b) yellow and green, and (c) blue and violet. It was found impossible to divide the spectrum in more regions because of its size and the amounts of serums necessary for accurate titrations.

After the exposures, titrations were made by using an antisheep amboceptor cell system. Two units of amboceptor and 5% cell suspensions were used, the total volume of each being 0.25 c c. Complement was employed in quantities of 0.05, 0.075, 0.125, 0.150, 0.175, 0.200, 0.250, and 0.300 of 1 c c of a 10% dilution. Saline was added to balance. The time of incubation was 30 minutes at 37 C. in a water bath.

Table 1 presents the results of a series of exposures to the entire spectrum and for various periods of time. The serum used in these tests was undiluted.

The results of these experiments indicate clearly that 3 hours' exposure had practically no effect on the serum, while from 6 hours on the inhibitory action becomes visible and gradually increasing until the complement is practically destroyed after an exposure of 29 hours. Several exposures were made for periods less than 3 hours but with no effect.

Table 2 gives the results of exposures of diluted serum (10%) and also of dried serum. The diluted serum in this experiment was

the same as the one used in the first undiluted (3 hours) experiment. Whereas in the first experiment only 0.125 of 1 cc of the complement was necessary to produce complete hemolysis, the same serum exposed in a diluted state required 0.175 of 1 cc to produce complete hemolysis (3 hours). Nineteen hours' exposures entirely inhibited the activating power of 0.125 of 1 cc of the undiluted serum, and 0.25 of 1 cc of the same serum diluted was required to give slight hemolysis. It seems

TABLE 1
EFFECT OF THE ENTIRE VISIBLE SPECTRUM ON COMPLEMENT

Amounts of Complement Used	3 Hours		6 Hours		19 Hours		29 Hours	
	Exposed	Dark	Exposed	Dark	Exposed	Dark	Exposed	Dark
0.05 c c	NH	SH	NH	NH	NH	NH	NH	NH
0.075 c c	SH	ACH	NH	MH	NH	TH	NH	NH
0.100 c c	ACH	CH	NH	CH	NH	SH	NH	NH
0.125 c c	CH	CH	NH	CH	NH	MH	NH	SH
0.150 c c	CH	CH	TH	CH	TH	ACH	NH	MH
0.175 c c	CH	CH	SH	CH	SH	CH	NH	CH
0.200 c c	CH	CH	MH	CH	MH	CH	NH	CH
0.250 c c	CH	CH	MH	CH	ACH	CH	NH	CH
0.300 c c	CH	CH	CH	CH	SH	CH

CH, complete hemolysis; ACH, almost complete hemolysis; MH, marked hemolysis; SH, slight hemolysis; TH, trace hemolysis; NH, no hemolysis.

TABLE 2
EFFECT OF THE ENTIRE VISIBLE SPECTRUM ON DILUTED AND UNDILUTED COMPLEMENT

Amount of Complement Used	Diluted				Dried			
	3 Hours		19 Hours		24 Hours		48 Hours	
	Exposed	Dark	Exposed	Dark	Exposed	Dark	Exposed	Dark
0.05 c c	NH	SH	NH	NH	NH	NH	SH	MH
0.075 c c	SH	MH	NH	SH	NH	NH	MH	CH
0.100 c c	MH	ACH	NH	MH	SH	SH	ACH	CH
0.125 c c	MH	CH	NH	CH	MH	MH	CH	CH
0.150 c c	ACH	CH	NH	CH	ACH	ACH	CH	CH
0.175 c c	CH	CH	NH	CH	CH	CH	CH	CH
0.200 c c	CH	CH	TH	CH	CH	CH	CH	CH
0.250 c c	CH	CH	SH	CH	CH	CH	CH	CH
0.300 c c	CH	CH						

evident, therefore, that diluted serum is distinctly more sensitive to the action of light than undiluted serum.

Dried serum, on the other hand, seems to be much more resistant to the action of these rays. Forty-eight hours' exposure only slightly modified the complementing power of the serums used, while 6 hour exposures were sufficient to attenuate normal undried serums. In table 3 the comparative effect of the rays of larger wave lengths to those of shorter wave lengths are tabulated.

This experiment shows clearly that the green-blue-violet end of the spectrum is considerably more active in the destruction of complement than the red-orange and yellow end. The experiment further demonstrates that the red-orange and yellow rays have some inhibiting action on complement. There was a demonstrable delay of hemolysis, and, as shown, 0.152 of 1 c c of exposed complement was necessary to secure complete hemolysis, while 0.075 of 1 c c of the same nonexposed serum sufficed.

TABLE 3

THE ACTION OF THE RAYS OF LARGER WAVE LENGTHS COMPARED TO THAT OF THE RAYS OF THE SHORTER WAVE LENGTHS ON COMPLEMENT. (EXPOSURE 19 HOURS)

Amount of Complement	Reds, Orange, Yellow Regions		Green, Blue, Violet Regions	
	Exposed	Dark	Exposed	Dark
0.05 c c	MH	MH	NH	NH
0.075 c c	MH	CH	NH	MH
0.100 c c	ACH	CH	NH	ACH
0.125 c c	CH	CH	NH	CH
0.150 c c	CH	CH	NH	CH
0.175 c c	CH	CH	TH	CH
0.200 c c	CH	CH	SH	CH
0.250 c c	CH	CH	MH	CH

TABLE 4

DIVISION OF SOLAR SPECTRUM INTO THREE REGIONS AND THE EFFECT OF EACH REGION ON COMPLEMENT

Amount of Complement	2.5 Hours		Control		4.5 Hours		Control	
	Infra-red Red	Yellow-green	Blue-violet	Dark	Infra-red Red	Yellow-green	Blue-violet	Dark
0.050 c c	NH	NH	NH	MH	NH	NH	NH	SH
0.100 c c	NH	NH	NH	ACH	TH	TH	NH	CH
0.125 c c	MH	SH	SH	CH	MH	MH	SH	CH
0.150 c c	CH	CH	CH	CH	ACH	ACH	MH	CH
0.175 c c	CH	CH	CH	CH	CH	CH	CH	CH
0.200 c c	CH	CH	CH	CH	CH	CH	CH	CH
0.250 c c	CH	CH	CH	CH	CH	CH	CH	CH

In the following experiment the solar spectrum has been divided into three zones, the infra-reds and red, the yellow-green and the blue-violet zones. The light of the sun is rich in infra-reds and red rays, and it is for that reason that the study of the solar spectrum is of importance to determine the direct effect of these rays (table 4).

From the results, it can be seen that the red and infra-red rays have a slight inhibitory action on complement. The effect of these rays was mainly one of delay of reaction when compared with the control not exposed half of the serum. As stated, the effects of the solar spectrum deserve further study.

SUMMARY

The effect of the visible spectrum on complement is one of inhibition. Exposed serums do not produce hemolysis in the same unit of time as serums kept in darkness and may be even greatly reduced in their complementary action if the exposures have been sufficiently long. Hemolysis is distinctly delayed, and if the exposures have been made for a long enough period the effect produced is lasting. Red and infra-red rays delay the activating action of the complement. At the violet end of the spectrum inhibition is marked. Dried serums are more resistant to the action of light and only prolonged exposures are able to weaken the complement, while diluted serums are much less resistant. The decrease of complementary power after an exposure of 6 hours, for instance, does not continue after the radiation is terminated. The reaction, then, is not continuous. This confirms the work of Bovie and Brooks and of Huber⁸ for exposures of rennin to ultraviolet rays. With continued exposure, however, attenuation markedly increases as pointed out by Lundberg.⁶ The nature of light action on complement is still problematical. It appears as if it is a purely chemical change. Abelin and Stiner² state the action of light (ultraviolet) to be one of molecular change and give as an example the change of the poisonous yellow-phosphorus into nonpoisonous red-phosphorous. The action does not take place with absorption. Bovie showed that 20% of the cells protect complement from destruction, and Soret⁹ has shown that most proteins exhibit an absorption band in the ultraviolet end of the spectrum and that solutions of tyrosin exhibit this phenomenon most markedly. Only tyrosin and phenylalanin seem to show this property as reported by Kober.¹¹ Whatever the nature of photosensitiveness of complement, light inhibits and destroys complement, and its action can be antagonized by the use of a member of the aromatic-amino acid series, as shown most recently by McCoy, Hill and Schmidt. It remains for future work to determine the exact nature of this effect.

⁶ Arch. f. Hyg., 1905, 54, p. 53.

⁹ Arch. d. Sc. phys. et nat. Geneva, 1878, p. 322; 1883, p. 194.

¹⁰ Jour. Biol. Chem., 1915, 22, p. 433.

EFFECT OF HEMORRHAGE ON COMPLEMENT OF BLOOD

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The effects of hemorrhage on the cellular elements of the blood have been fairly well established. According to Drinker,¹ within 10 minutes after hemorrhage, the leukocyte count begins to rise, and reaches its height within a few hours. Following a single large hemorrhage it may remain elevated for a week. Next comes an increase in platelets, which begins to be manifest during the first 24 hours, and reaches a maximum within the first few days. The young red forms are slower to appear; and the red count is made up much more slowly than either of the others, depending on the size and duration of the hemorrhages. The hemoglobin content is the last to return to normal. The anemia following chronic conditions of loss of blood according to Lee and Minot² shows less tendency to marked leukocytosis, less tendency to marked increase in immature forms, but aplasia of the blood forming organs never results from hemorrhage. As pointed out by Robertson and Bock,³ the fluid content of the blood is not fully made up until the third day; and hence the red count may continue to fall until that time, due to gradual dilution, unless fluids are forced on the patient.

On the other hand, the relation between these changes and complementing power of the blood have not been studied. Coca⁴ states that the regeneration of complement after hemorrhage, when the bulk of the lost blood is replaced with inactivated serum and washed corpuscles, is more rapid than can be accounted for, on the grounds of the addition of complement to the blood by the lymph, the latter being supposed to have the same titer as the normal blood. It, therefore, seemed to us desirable to ascertain the changes induced in the complement of the blood by profound and multiple hemorrhages and subsequent blood regeneration; and what, if any, were the relations of this change to changes in the cellular elements.

Received for publication June 12, 1922.

¹ Oxford Medicine, 1920, 2, p. 509.

² Nelson Loose Leaf Medicine, 1920, 4, p. 17.

³ Jour. Exper. Med., 1919, 29, p. 155.

⁴ Ztschr. f. Immunitätsf., 1914, 20, p. 622.

Guinea-pigs were used on account of the known stability of the complement titer in this animal. The normal titer was established in all cases, except in the last series, by 3 preliminary titrations and 2 blood counts. The ear veins were found satisfactory for occasional bleeding, but when repeated withdrawals of blood in amounts of 1.5 cc or more, coupled with additional punctures for blood counts, were necessary, heart puncture was found to be the only practical method.

The counting of platelets was best accomplished in a solution of 3.8% sodium citrate, plus 0.2% formaldehyd and enough brilliant cresyl blue 0.1% to stain the platelets a deep blue. The solution was found to become hemolytic (?) unless made fresh every few days, and was always filtered immediately before using. The methods of Pratt,⁵ Wright and Kinnicutt,⁶ and Buckman and Hallisey⁷ were tried and discarded. The method used allowed the counting of red cells and platelets in the same preparation, the red count being checked from time to time with Hayem's solution. The procedure was as follows: A red-counting pipet was first filled with diluting fluid to the 0.5 mark; blood was drawn rapidly until the top of the column of fluids touched the 1 mark, when the pipet was again plunged into the diluting fluid. The resulting dilution was 1:200.

The serum for titration was obtained, as a rule, from 1.5 cc of blood and was diluted 1:20, in which dilution a variation of 2 tubes is not to be considered significant when a fresh hemolytic series had to be prepared each day. When, however, an interval of only a few hours allowed the use of the same hemolytic series, with identical pipet, etc., the variation of a single tube is to be noted. An excess of amboceptor (4 units) was used.

During the entire experiment the animals were fed on fresh vegetables, but were given no fluids to drink. To avoid the danger of a complicating infection the animals after the first pair were not tagged by piercing the ear.

The first series covered a period of 30 days up to the death of animal B, although the observations were somewhat irregular. Animal A was used again later, and observations on this animal covered 74 days and a total of 6 more or less severe hemorrhages. From repeated small hemorrhages the animals reached a stage of mild chronic anemia, as shown by the immature red forms and the slight reaction of

⁵ Jour. Am. Med. Assn., 1905, 45, p. 1919; 1906, 46, p. 1092.

⁶ Ibid., 1911, 56, p. 1457.

⁷ Ibid., 1921, 76, p. 427.

leukocytes and platelets following the last bleeding. The differential leukocyte count showed a progressive shift toward the mononuclear elements at the expense of the polymorphonuclear neutrophils. No constant or significant variation in complement was made out.

The second series reached a more profound anemia, the relation of the red count to complement titer being shown in chart I. Except for a slight drop in complement in the specimen taken 3 hours after the first hemorrhage, which was regained the next day, there were no

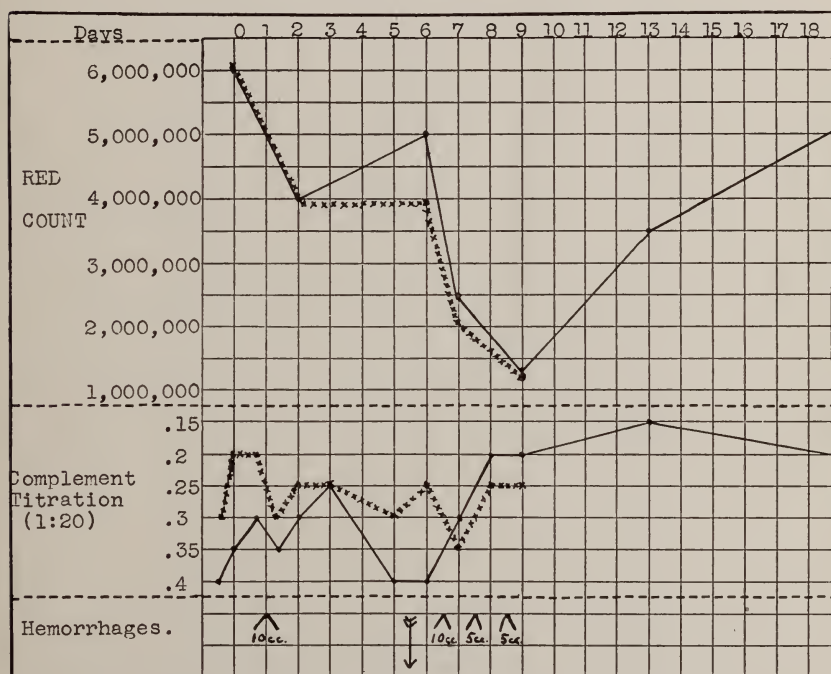


Chart I.—Relation of red count to complement titer in the second series of animals. The curve of animal C is indicated by the x x x line, that of animal D by the continuous line.

changes in titer attributable to hemorrhage. Animal C, which was pregnant when the experiment was undertaken, showed a somewhat irregular but generally weaker titer up to parturition; it is interesting to note that the titer promptly returned to what appears subsequently to be the normal level, and did so in spite of 3 successive hemorrhages. The behavior of leukocytes and platelets, both as to numerical and differential count, was similar to that of the preceding series, except for the appearance of a few myelocytes.

Animals E and F of series three showed characteristic cellular reactions following a single severe hemorrhage, but no significant changes in complement titer. In this experiment blood was tested one hour after hemorrhage. In contrast to the previous finding, the titer was unaltered.

Apparently no appreciable or constant variation in the titer of the blood can be made out 24 hours after a hemorrhage even of great

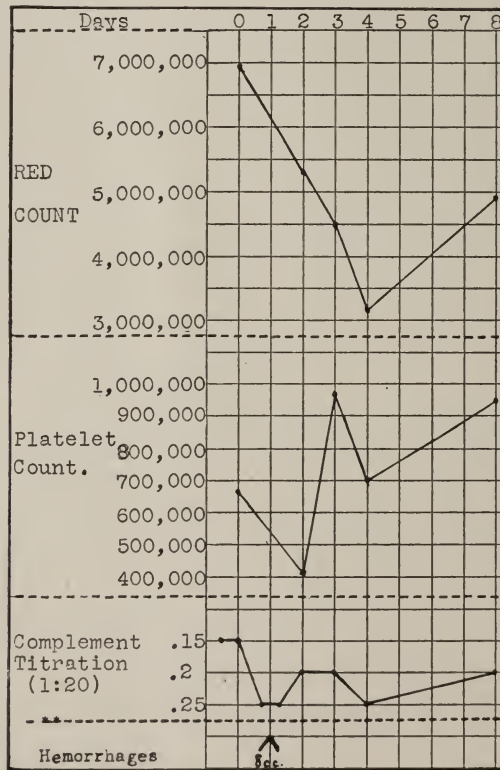


Chart 2.—Reaction of red cells and platelets in a typical animal.

severity. Neither the subsequent dilution nor any event in the course of regeneration of the cellular elements affects the titer. The reaction of red cells and platelets in a typical animal is shown in chart 2, in which dilution is seen not to be complete until the third day after a severe hemorrhage. The leukocyte counts are incomplete and the reactions insufficient to show a characteristic curve. That repeated hemorrhages reducing the red count to about one-sixth of normal and

prolonging the period of anemia have also no effect on the level of complement present in the blood, is also to be deduced from the several animals on which this was tried. The rise in titer following parturition is interesting but does not come within the scope of this paper.

The observation that the complementing power showed a distinct drop 3 hours after hemorrhage led us to add the following experiment:

Eight guinea-pigs, of which the red and platelet counts had been determined, were bled from 10 to 11 cc according to their weight,

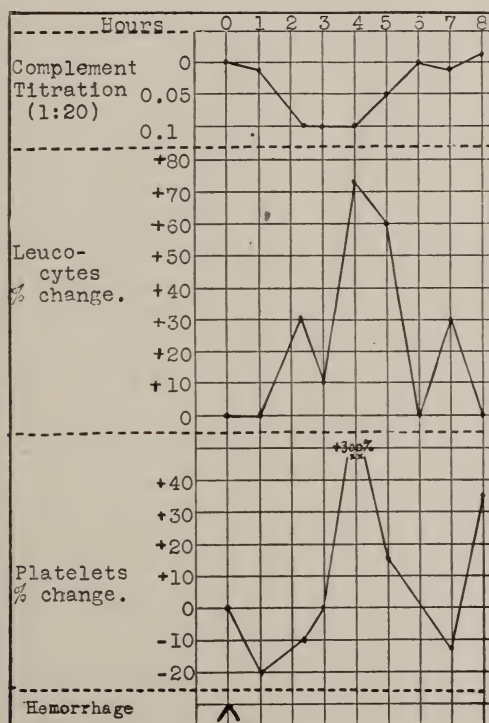


Chart 3.—Results in third experiment.

which ranged around 600 gm. The normal complement figure for each animal was obtained from this blood. Following this counts were made and blood titrated from 1 or more animals at intervals of 1 hour, so that in the end each animal had been used twice, besides the original hemorrhage. The greatest care was taken to make the results exactly comparable.

The result is best given in chart 3 in which the observations are combined and averaged. The leukocyte count will be seen to begin

to rise after 1 hour, while the platelet count shows an initial drop. As dilution is considered as beginning at once, this should mean an immediate production of leukocytes, which reaches a maximum within a few hours and then slows down. The platelets, on the other hand, begin their regeneration more slowly and show greater individual variation. Previous experience would indicate that the count does not remain above normal until after 24 hours, although production is undoubtedly taking place, as shown by the fluctuations in count, resulting in practically offsetting the rate of dilution.

The curve of complement shows the beginning of a drop after 1 hour (1 out of 4 animals), but after $2\frac{1}{2}$ hours there is a weakening of 2 tubes. This weakening was constant for all animals until the fifth hour when the titer began to increase, and by the sixth hour the complementing power seems to have returned almost constantly to normal. In reading these titrations the results were rendered difficult by the fact that in addition to the weakening, the end-point of complete hemolysis was rendered less distinct, and several tubes might show almost complete hemolysis before one could be called clear. This was a contrast to the sharp end-points of the normal titrations.

The origin of complement, then, is not in the cells of circulating blood. There appears to be no relation between the curve of complement regeneration and the reactions of the various cellular elements. That the tissue fluids first called on to make up the lost plasma are poor in complement would seem to be the reason for the initial drop. There is probably a reservoir or factory of complement, as yet unknown, whose response is fairly prompt and very accurate, since an overproduction comparable to that found in leukocytes and platelets is not seen. In using this terminology, it must not be supposed that anything is assumed regarding the nature of this substance, for we are not in a position to say that it is elaborated after any particular manner.

SUMMARY

Complement has a regular curve of decrease and regeneration following a single severe hemorrhage.

It is unaffected by subsequent dilution of the blood by the body fluids.

The curve does not run parallel to that of any of the cellular elements circulating in the blood following hemorrhage, nor is there reason to believe that any of these cells is a factor in the regeneration of complement.

The initial decrease is due to dilution of the blood, which decrease is soon overcome and restored to normal by an influx of complement from some, as yet unknown, source.

Repeated hemorrhages with extreme degrees of anemia and subsequent recovery do not alter the level of complement.

THE RELATION OF REDUCTION OF LEUKOCYTES AND PLATELETS TO COMPLEMENTING POWER OF SERUM

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The rôle of the leukocytes in the production of complement has been a matter of considerable study and dispute. The greater bulk of work on the subject was done when no clear differentiation had been made between amboceptor and complement, and by various methods of making extracts of white blood corpuscles. In vivo experiments have not received sufficient consideration, and it is with this point in view that we have attempted to reduce the number of the circulating leukocytes to ascertain whether or not a parallel reduction of complement could be demonstrated.

Historical Survey.—The hypothesis that complement might originate in the leukocytes was first advanced by Metchnikoff.¹ The original observation of Metchnikoff, however, was limited to the bactericidal substances contained in the polymorphonuclears. Following the work of Tarassewitch,² Shibayama,³ and of Klein,⁴ who showed that extracts of spleen, pancreas, omentum and lymph glands were endowed with hemolytic properties, Metchnikoff conceived the idea that the bacteriolytic complement was contained in the polymorphonuclear leukocytes (microcytase) and that the hemolytic complement was a different body and contained in macrophages (macrocytase). Metchnikoff received the support of Wassermann,⁵ Ascoli and Riva,⁶ Hahn⁷ and others. It must be emphasized that in much of the earlier work insufficient attention was paid to carefully separating cells from serum. Some investigators (Wassermann, Ascoli and Riva) used an anticomplement produced by injecting washed leukocytes in rabbits, the resulting immune serum having an inhibiting action on hemolysis. However, similar observations were made by Donath and Landsteiner⁸ who used red blood corpuscles, lymphnodes, milk, etc. The phenomenon, therefore, was not specific and not due to an anticomplement, as originally conceived. Prior to the latter studies, Schattenfroh⁹ and Moxter¹⁰ had shown that the bactericidal substances of polymorphonuclear leukocytes did not act as complement in hemolysis, and following their work Lambotte

Received for publication June 12, 1922.

¹ Ann. l'Inst. Pasteur, 1899, 13, p. 737.

² Ibid., 1902, 16, p. 127.

³ Centralbl. f. Bakteriöl., I, O., 1902, 30, p. 760.

⁴ Wien. klin. Wchnschr., 1901, 14, p. 1309.

⁵ Ztschr. f. Hyg. u. Infektionskr., 1901, 37, p. 173.

⁶ München. med. Wchnschr., 1901, 48, p. 1343.

⁷ Arch. f. Hyg., 1895, 25, p. 105.

⁸ Ztschr. f. Hyg. u. Infektionskr., 1903, 43, p. 552.

⁹ Arch. f. Hyg., 1897, 31, p. 1.

¹⁰ Deutsch. med. Wchnschr., 1899, 25, p. 687.

and Stiennon,¹¹ Petterson,¹² Schneider,¹³ Gruber,¹⁴ Neufeld,¹⁵ Kling,¹⁶ Zinsser,¹⁷ Dick,¹⁸ and recently Gengou¹⁹ have made similar demonstrations. From these studies we have learned that leukocyte extracts resist a temperature of 60 C., a fact that in itself separates them from complement. Gengou, furthermore, found that these extracts do not contain mid- or end-piece of the complement. So far we have been able to find, Lippmann and Plesch²⁰ are the only investigators who studied the problem in vivo. They found that the regeneration of complement in guinea-pigs occurs after the destruction of the bone marrow and the leukocytes of the blood by injections of large quantities of thorium X. In our series of experiments we have used benzene and in counting the leukocytes also counted the blood platelets to establish their relation to complements.

Method.—Equal parts of benzene and olive oil were mixed and injected subcutaneously in doses of 1 c.c. benzene per kilo of animal. Rabbits were selected because of their known susceptibility to benzene and because their serum contains a fair amount of complement. Guinea-pigs were found to be resistant to benzene; double the amount necessary for the rabbit fails to reduce the circulating leukocytes of the guinea-pig. The benzene, furthermore, produces extensive necrosis in the guinea-pig and secondary infection readily occurs. Following the injections of the benzene olive oil mixture into rabbits, the site of injection was carefully massaged to allow a wide area of absorption. Three or 4 injections sufficed to reduce the circulating leukocytes to a figure below 1,000 per c. mm. In the first series of experiments the platelet counts were made by allowing blood to flow from the marginal vein through a needle into a syringe barrel containing 2% sodium citrate. The piston was then introduced and the barrel inverted a few times. Smears were made on glass slides and stained with Wright's stain. Comparative counts of platelets and red cells were then made. At least 40 fields covering the entire smear were counted and a separate count of erythrocytes made by the usual pipet counting chamber method. In the second series of experiments a brilliant cresyl blue citrate solution was used, and direct counts of platelets were made. The white count was made as usual.

The complement was titrated by diluting the serum with equal parts of Ringer-Tyrodé's solution. Four units of amboceptor were used and

¹¹ Centralbl. f. Bakteriöl., I, O., 1906, 40, p. 224.

¹² Ibid., 1905, 39, p. 423; 1908, 46, p. 405.

¹³ Arch. f. Hyg., 1909, 70, p. 40.

¹⁴ Cited by Sachs in Kraus and Levaditi, Handbuch der Technik. u. Methodik. der Immunitätsforschung.

¹⁵ Arb. a. d. Kais. Gesundh. Anat., 1908, 28, p. 198.

¹⁶ Ztschr. f. Immunitätsf. 1910, 7, p. 1.

¹⁷ Infection and Resistance, 1918.

¹⁸ Jour. Infect. Dis., 1913, 12, p. 111.

¹⁹ Ann. l'Inst. Pasteur, 1921, 35, p. 497.

²⁰ Ztschr. f. Immunitätsf., 1913, 17, p. 548.

a carefully prepared 5% sheep cell suspension. The sheep blood was obtained from an ordinary laboratory sheep. The following were the results of the experiments:

RESULTS

First Series.—Rabbit 37 (weight 1.7 kg.); on Nov. 12, 15, 18 and 22, the average complement titer was 0.2 cc (1:2 dilution). The erythrocytes numbered 4,740,000, the platelets 853,200 and the leukocytes 8,600. Nov. 23, 24 and 25 this animal received 3 injections of 3.4 cc benzene olive oil mixture. Nov. 28, the leukocytes numbered 440, and on Nov. 29, the platelets numbered 70,000. The complement titer remained as before at 0.2 cc. The animal succumbed on Nov. 29. The bonemarrow of this animal was bloody and edematous; there was marked engorgement of blood vessels with numerous hemorrhages and relatively few myeloid cells. Scattered giant cells were seen. The spleen showed marked engorgement of sinuses, numerous hemorrhages; the blood pigment was in places intracellular, in places extracellular; malpighian bodies in places were smaller than the average. The liver showed no appreciable abnormalities.

Rabbit 36, weight 1.8 kg., on Nov. 12, 15, 18, and 22, the animal had an average complement titer of 0.21 cc (1:2 dilution). The erythrocytes numbered 4,964,000, the platelets 397,120 and the leukocytes 11,120. Nov. 23, 24 and 25, three injections of 3.6 cc of benzene olive oil mixture were made. Nov. 27, the white count dropped to 540 cells per c.mm., the platelets numbered 616,000. Nov. 27 and 28, the complement titer was 0.2 cc (1:2 dilution). This animal died on Nov. 28. The bone marrow was also bloody, edematous; there was marked engorgement of blood vessels and relatively few myeloid cells present. In the spleen the sinuses were moderately engorged; the malpighian bodies were smaller, the trabeculae conspicuous. The liver was normal.

Rabbit 23 (weight 2.2 kg.), on Nov. 15, 18 and 22 had an average complement titer of 0.25 cc (1:2 dilution). The erythrocytes numbered 6,316,000, the platelets 536,800 and the leukocytes 8,740. Nov. 23, 24, 25 and 27, four injections each of 4.4 cc benzene olive oil mixture were made. On Nov. 30, the white count dropped to 1000, and on Dec. 1, it was 800. On Dec. 1, the platelets numbered 50,000. Throughout the experiment the complement titer of this animal remained 0.25 cc. The bonemarrow on section showed almost contiguous engorgement of blood vessels, in places the vessel walls ruptured; there were few myeloid cells present. The sinuses in the spleen were engorged with blood; there was much intercellular and extracellular altered pigment; the malpighian bodies were hardly recognizable and in places appeared smaller than the average. The liver showed areas of scarring, especially marked about the large vessels. In general, there was no abnormality of the parenchyma.

Rabbit 30; control; weight, 1.6 kg.; on Nov. 12, 15, 18 and 22, the complement titer averaged 0.21 cc (1:2 dilution). The erythrocytes numbered 6,264,000; the platelets 500,000 and the leukocytes 7,305. This animal received 3 injections of olive oil in doses of 1.6 cc on Nov. 23, 24, 25. On Nov. 28, the complement titer was 0.25 cc and on Dec. 1, also 0.25 cc. On Nov. 29, the white count was 13,280, and on Dec. 1, 8700. The platelet count on Dec. 1 was 700,000. The animal survived.

Rabbit 39; control; weight, 2.6 kg.; Nov. 22 and 23 the complement titer averaged 0.2 cc (1:2 dilution). The erythrocyte count was 5,076,000; the platelets numbered 500,000 and the white cells 9,720. Nov. 23, 24 and 25, this animal received each day 2.6 cc of olive oil. On Nov. 28 and Dec. 1, the

complement titer remained at 0.2 cc; the platelets, respectively, 800,000 and 900,000; and the white cells fell from 15,560, on Nov. 28 to 11,240, on Dec. 1. The animal survived.

Rabbit 10; control; weight, 1.6 kg.; on Nov. 12, 15, 18 and 22, the complement titer was 0.2 cc (1:2 dilution). The erythrocytes numbered 5,644,000, the platelets 400,000 and the white corpuscles 11,895. This animal received no injections. On Nov. 28, 29 and Dec. 1, the complement titer remained as before. On Nov. 28, the red cells numbered 5,944,000; the platelets 540,000; and the white cells 7,680.

Second Series.—In this series the same experiments were repeated. The only difference between the first and second series was the method of platelet count as indicated in the foregoing.

Rabbit 290; weight, 1.8 kg.; on April 12, 13 and 14, the complement titer of this animal averaged 0.23 cc (1:2 dilution), the platelets 784,000, the white cells 15,200. The rabbit received 3.6 cc benzene olive oil mixture daily on April 15, 16, 17 and 18. On April 19, the white count dropped to 1400; the platelets were 752,000. The complement on April 19 was 0.2 cc and April 20, 0.15 cc. The animal died on April 23. The pathologic picture was the same as in the other rabbits that died of the poison.

Rabbit 293; weight, 1.6 kg; April 12, 14 and 15, the complement titer of this animal was 0.18 cc (1:2 dilution). The platelets numbered 636,000 and the whites 17,770. On April 15, 16, 17 and 18 the animal received 4 doses each of 3.2 cc benzene olive oil mixture. The white count dropped on April 19 to 500; the platelets, however, were slightly higher, namely, 754,000. On April 20, the platelets were 664,000; the white cells 580. The complement on April 19 was 0.2, and on April 21, 0.15 cc. On this day the animal died, and the postmortem picture was the same as in the other animals.

Rabbit 294; control; weight, 1.6 kg.; on April 12, 14 and 15 the complement of this animal titered 0.25 cc (1:2 dilution); the platelets numbered 368,000; the white count was 17,560. On April 15, 16, 17 and 18, the animal received 4 injections of 1.6 cc olive oil. On April 19 and 21, the complement titer did not change. On April 19, the platelets numbered 416,000 and the leukocytes 24,000, which count dropped to 9,000 April 20. The animal remained normal.

SUMMARY

From these experiments it appears that the rabbit readily responds to the action of benzene. In every instance the leukocytes were brought down to a figure below 1,000. The same is not true for the platelets. Some animals had a slight increase in platelets, an observation already made by Duke.²¹ The control animals that received olive oil alone showed a transient leukocytosis. The complement titer of all the animals remained within normal variations so that it is fair to conclude that the reduction of the leukocytes and platelets in the circulating bloodstream does not go parallel with a change in the complement titer of the blood serum of the treated animals. Since the bone-marrow and spleen exhibit serious deterioration as the result of the benzene treatment, it is apparent that these tissues are not concerned in

²¹ Jour. Am. Med. Assn., 1915, 65, p. 1600.

any important way in the maintenance of the complement of the serum. Whether the diminution of leukocytes is only apparent, or, as suggested by Pappenheim,²² they accumulate in the dilated capillaries of the liver and other organs is, at present, difficult to state. Our observations indicate that they do not accumulate in the liver. The condition of the marrow strongly suggests that there is an actual reduction in the number of circulating leukocytes, and most modern studies point toward an absolute reduction. Antibodies are reduced following benzene injection, as pointed out by Hektoen.²³ On the contrary, the complementing power of the blood serum is not altered as the result of subcutaneous injection of benzene.

²² Wien. klin. Wchnschr., 1913, 26, p. 48.

²³ Jour. Infect. Dis., 1916, 19, p. 69.

ON THE GROUP SPECIFICNESS OF ANTIBODIES IN ANTISTREPTOCOCCUS SERUM

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In previous articles I¹ have shown that sheep immunized with hemolytic streptococci from scarlet fever and erysipelas, respectively, produced specific opsonins and agglutinins. The results of absorption tests also indicated that the streptococci from these diseases belonged to distinct biologic groups. Protection experiments with the streptococci from scarlet fever gave additional weight to this inference in respect to the scarlatinal group. Bliss² and Gordon³ reached similar conclusions concerning streptococci from scarlet fever, by means of agglutination tests with immune rabbit serum.

TECHNICAL

In my experiments the animals were immunized first with heat-killed organisms and later with living. The cocci were grown in ascitic dextrose broth for 24 hours, centrifuged and suspended in salt solution and then heated at 60 C. for one hour. When living cocci were injected, they were grown in goat blood agar slants for 24 hours and the growth washed off in salt solution and injected intravenously. The killed organisms were first injected subcutaneously into rabbits and later intravenously. All of the injections into the sheep were intravenous. As a rule, the animals were inoculated every day for 4 days with increasing doses followed by a rest of 4 days. This was repeated 2 or 3 times, and then weekly doses were given for a month or more. The growth on 1 blood agar slant was the maximum dose of living organisms for rabbits, the growth on 3 for sheep. Larger doses stopped antibody production. The animals were generally bled 4 days after an injection.

For the opsonic experiments, the cocci were grown on goat blood agar for 24 hours and suspended in salt solution. Normal leukocytes, sheep with sheep serum and human with rabbit serum collected in 0.2%

Received for publication, June 26, 1922.

¹ Jour. Am. Med. Assn., 1920, 74, p. 1386, and 75, p. 1339.

² Bull. Johns Hopkins Hosp., 1920, 31, p. 173.

³ Brit. Med. Jour., 1921, 1, p. 632.

sodium citrate solution and washed once in salt solution, were used. The serum, normal and immune, was heated for one-half hour at 56 C. to remove the thermolabile element, and then diluted with salt solution. The mixtures of diluted serum leukocytes and coccal suspensions, equal parts, were incubated for 25 minutes, smears stained with carbol thionin, 100 polymorphonuclear leukocytes counted, and the number of cells taking part in phagocytosis noted. The point of opsonic extinction was determined by finding the dilution at which opsonification ceased.

In the agglutination tests cocci were grown from 24 to 48 hours in ascitic dextrose broth, 1 part of ascitic fluid to 4 parts of 1 per cent. dextrose broth P_H 7.8. The dextrose broth was made with Fairchild's cultural peptone. The cultures were centrifuged, the supernatant fluid removed and the cocci suspended in salt solution. At first the serum dilutions were made with plain broth but later salt solution was used, the final dilutions running from 1:20 to 1:800, an equal part of bacterial suspension being added to each tube of diluted serum. Tubes containing cocci in broth or salt without serum and also mixtures of normal serum at the same dilutions as the immune serum were included in each test. The immunizing streptococcus was tested in each experiment to serve as a standard. The mixtures were incubated from 1 to 3 hours at 55 C. Stored serum sometimes agglutinated more slowly than others, but as a rule agglutination was complete at the end of 1 hour. Ascitic fluids of low specific gravity, say 1.008, with a P_H 7.8 are particularly suitable for this work. A specific gravity of 1.020 in one instance caused spontaneous clumping of all the strains of streptococci. Some lots of broth caused spontaneous clumping, the reason for which was not determined.

The absorption tests were made in different ways. The organisms were grown in ascitic dextrose broth for 24 hours, centrifuged, suspended in salt solution, heated at 60 C. for 1 hour, centrifuged and the serum to be absorbed added to this, in order to dilute the serum as little as possible. Care was taken that the absorbed serum was not diluted in higher dilutions than the unabsorbed. At first the suspended cocci and serum were incubated 2 hours at 37 C., refrigerated for 24 hours, and finally centrifuged, the supernatant serum being removed. This process was repeated 3 or 4 times. Later the mixtures were incubated over night at 37 C. and this repeated if the serum was still found to agglutinate the immunizing coccus. Incubation at 55 C. for varying periods was less successful.

SHEEP

The serum from the sheep immunized for 5 months with the streptococcus from scarlet fever, specifically agglutinated and opsonified hemolytic streptococci from scarlet fever, after 11 months' storage in the icebox. It was necessary, however, to activate the serum with fresh sheep serum (one part in 9 of immune serum) to demonstrate the opsonins.

This sheep, after 10 months' immunization, produced agglutinins and opsonins for hemolytic streptococci from sources other than scarlet fever. It was found difficult to absorb these nonspecific agglutinins. Absorption was repeated 4 times. Absorption with a streptococcus from erysipelas and 1 from acute tonsillitis failed to remove the agglutinins, except in one instance in which the tonsillitis streptococcus removed the agglutinins for its own and 2 scarlet fever streptococcus strains. On the other hand, the immunizing scarlet fever streptococcus in every case absorbed antibodies for the heterologous but rarely the homologous scarlet fever streptococcus, and never those from other sources. No explanation is offered for these results. The experiments were repeated several times.

Another sheep was now immunized as before with a hemolytic streptococcus from the throat of a patient with an acute case of scarlet fever. This streptococcus was agglutinated and opsonified in high dilutions by the stored immune sheep serum, specific for scarlet fever. After 3 weeks' immunization the serum agglutinated 4 strains of streptococci from scarlet fever, but not 5 strains from other sources. After being stored in the icebox for 2 days it lost its specificity, agglutinating nonscarlatinal strains in as high a dilution as scarlatinal. On absorption with the homologous coccus over night at 37 C., the agglutinins for 4 scarlet fever strains were absorbed, but not 4 from other sources, which would indicate that the serum was specific but contained minor agglutinins in large amounts. At the next bleeding, one week later, the serum specifically agglutinated streptococci from scarlet fever, but the following day it agglutinated some nonscarlatinal strains. The third bleeding, one week later, gave serum which agglutinated 8 strains of scarlet fever streptococci, but not 14 strains from other sources. This specimen lost most of its agglutinating power after two days in the icebox, but did not show any indication of nonspecificity. The serum from the next bleeding, one week later, agglutinated streptococci from all sources in the same dilution as the

homologous scarlet fever strain 1:200. Four days later fresh serum agglutinated the homologous strain at a dilution of 1:4,000, one scarlet fever strain at 1:36,000, a nonscarlatinal strain at 1:20. The following days this serum agglutinated the immunizing strain at only 1:20. No agglutinins were demonstrable after the next injection, 8 weeks after the first. The serum was examined as soon as drawn, and after a few weeks standing in the icebox, to determine whether an inhibiting substance as described by Coca and Kelly⁴ was present. None was demonstrated. Three days following the next inoculation agglutinins were present in dilutions as high as 1:2,400 for streptococci from scarlet fever, but not for those from other sources. This serum agglutinated the homologous streptococcus in a dilution of 1:80 only, and these only after 3 hours' incubation. This specimen of serum remained specific, but after 3 days in the icebox agglutinated no strain in a higher dilution than 1:20. No agglutinins could be demonstrated after the next inoculation.

A sheep immunized with a hemolytic streptococcus from erysipelas produced agglutinins and opsonins specific for streptococci from erysipelas for 4 months, then ceased to produce agglutinins, although opsonification was a marked property of the serum. No agglutinins were demonstrated for 4 months, then some were produced in a dilution of 1:50 for streptococci from erysipelas and also those from other sources. Absorption experiments were made with the homologous erysipelas streptococcus, a scarlet fever and tonsillitis streptococcus and a staphylococcus albus. The serum was absorbed over night at 37 C. for 2 successive nights as one absorption did not remove the agglutinins for the homologous coccus. The agglutinins were removed by all 3 strains of streptococci but not by the staphylococcus.

Another sheep was immunized for 2 months with a hemolytic streptococcus from tonsillitis. Neither agglutinins nor opsonins were produced for the immunizing coccus except occasionally agglutinins could be demonstrated at a dilution of 1:20. When agglutinins were present they were not specific, but were found only in very low dilutions.

Hooker and Anderson⁵ call attention to the great importance of taking into consideration the individuality of the animal in studies of antibody specificity. They showed that only a few rabbits produced specific antibodies for human erythrocytes. In some of their experi-

⁴ Jour. Immunol., 1921, 6, p. 87.

⁵ Jour. Immunol., 1921, 6, p. 419.

ments the specificity for iso-agglutinative corpuscular groups entirely disappeared after the second and third series of injections, but remained unchanged for over 4 months in the immune serum obtained from the first bleeding. In other rabbits the specificity was not impaired by subsequent injections.

RABBITS

Hamilton and Havens,⁶ Dochez, Avery and Lancefield,⁷ Bliss² and Gordon³ have produced specific hemolytic streptococcus agglutinins in rabbits. Dochez and his co-workers observed, however, that rabbits varied a good deal, some showing a wide range of cross agglutination and their serum was less specific than that of sheep. Evans⁸ and Browning and Wilson,⁹ found that apparently only certain rabbits produced antibodies.

First I immunized 6 rabbits with 2 strains of hemolytic streptococci from scarlet fever, 1 from tonsillitis and 1 from sinusitis. Agglutinins were readily produced, but no specificity could be demonstrated. Opsonins were present in low dilution and showed no specificity. The agglutinins were absorbed as thoroughly by one streptococcus as by another, streptococci from scarlet fever, erysipelas, sinusitis and tonsillitis being used. These results would indicate that common agglutinins only were produced in the rabbits immunized in this case.

Immunization with few and small doses: As it has been shown by various observers (Friedberger and Dorner,¹⁰ Lake, Osborne and Wells,¹¹ Butterfield and Neill,¹² Hooker and Anderson⁵) that animals may develop a serum of higher specificity after a few small doses and that increased polyvalency develops after further injections, fewer and somewhat smaller doses were tried in 5 rabbits. Hemolytic streptococci isolated from the throats of patients in the early stages of scarlet fever were used for immunization. The cocci were grown in 10 c c of ascitic dextrose broth for 24 hours, centrifuged, suspended in salt solution and heated at 56 C. for 30 minutes. This suspension was either injected intravenously in 1 dose or in a few instances in 3 doses on the same day. After 2 weeks small numbers of living cocci (one eighth of the growth on a blood agar slant) were injected intravenously

⁶ Jour. Am. Med. Assn., 1919, 72, p. 272.

⁷ Jour. Exper. Med., 1919, 30, p. 179.

⁸ U. S. Hyg. Lab. Bull., 1920, No. 124, p. 56.

⁹ Jour. Immunol., 1920, 5, p. 417.

¹⁰ Centralbl. f. Bakteriologie, O, I., 1905, 38, p. 544.

¹¹ Jour. Infect. Dis., 1914, 14., p. 364.

¹² U. S. Hyg. Lab. Bull., 1920, No. 124, p. 15.

every week or two to keep up antibody production. One rabbit was injected with cocci killed by suspending them in a saturated glucose solution over night at 37 C., centrifuging and replacing the glucose solution with salt.

Two rabbits produced no agglutinins. About one week after the first injection, the other rabbits showed specific agglutinins in their serum in dilutions from 1:160-1:1,280, but lost their agglutinating power in a few days on standing in the icebox. The heterologous scarlet fever streptococci were generally agglutinated in as high dilutions as the immunizing coccus. After receiving living organisms, the rabbits sometimes produced specific agglutinins in higher dilutions for varying periods, one as long as 4 months. Further injections have so far produced nonspecific serums. The serum of one rabbit remained specific for 3 months in the icebox. The serum of the rabbit immunized with glucose-killed streptococci agglutinated scarlatinal streptococci in higher dilutions (1:10,000) than the others. This may have been accidental but is in accord with experiments made by Dr. Weaver and myself,¹³ which showed that greater antibody production and protection was obtained after immunization with galactose-killed streptococci than by heat-killed streptococci.

For the agglutination tests the streptococci were now grown in phosphate broth, the formula used by Havens and Taylor.¹⁴ Meat infusion broth was made with distilled water, to which was added 1% peptone, 1% disodium phosphate and 0.5% glucose. It was adjusted to P_H 8-8.2. Tubes containing 10 c.c. of the broth were autoclaved at 10 pounds for 10 minutes. The organisms grew profusely in this medium, producing, as a rule, a perfectly homogeneous suspension. Occasionally it was necessary to transfer a culture several times in this broth in order to get rid of spontaneous clumping. The agglutination experiments were made as before except that the serum was diluted with phosphate broth and the cultures in phosphate broth used for the suspensions. The specimens were incubated for 1 hour at 55 C. Each experiment contained strains of the immunizing and another known scarlatinal streptococcus and 2 strains of known heterologous hemolytic streptococci as controls.

The absorption tests were made according to the method of Gordon,³ the immune serum being saturated with the various strains of streptococci to be examined, the absorbed serums then being tested with the

¹³ Jour. Infect. Dis., 1908, 5, p. 599.

¹⁴ Am. J. Hygiene, 1921, 1, p. 311.

immunizing coccus, a strain which grew homogeneously and clumped readily with specific immune serum was selected for immunization. The organisms were grown in phosphate broth for 24 hours, centrifuged, the supernatant fluid removed and the immune serum, diluted 1:10 with broth, added to this very thick mass of cocci. As the different strains grew equally well, each tube contained approximately the same number of organisms. The tubes were incubated at 55 C. for 2 hours and centrifuged, and agglutination tests were made with the supernatant fluid. If the immunizing coccus did not absorb the agglutinins in this time, the specimens were kept in the icebox over night and the supernatant fluid added to more cocci as before. It was rarely necessary to repeat the absorption more than twice. Fresh serum appeared to absorb more readily than stored serum. Here again each experiment was controlled with phosphate broth without serum, normal serum, unabsorbed immune serum, and immune serum absorbed with the homologous and another known scarlatinal streptococci and 2 known heterologous hemolytic streptococci.

Seventy-five strains of hemolytic streptococci have been examined for agglutinins with specific immune rabbit serum. Thirty-nine of these strains, 22 scarlatinal and 17 heterologous, were used for absorption tests. Thirty-six cultures were isolated from the throats of patients in the early stages of scarlet fever or from patients with its complications: purulent rhinitis (3), otitis media (3), mastoiditis (1), cervical adenitis (2), lochia (1), wound (1), and burn (1). The discharge from the cervical abscess of one of the patients had persisted for 4 months. The 39 nonscarlatinal strains were taken from patients with acute tonsillitis among attendants in the Durand hospital, discharges from ear and cervical glands of diphtheria patients, otitis media, mastoiditis, smallpox pustule, paranephritic abscess, peritonsillar abscess and the infected finger of the nurse of a scarlet fever patient.

None of the cultures were agglutinated by normal rabbit serum.

All of the strains from the scarlet fever patients except 3 were agglutinated in high dilutions by the specific immune serum. One of these was a mannite fermenter. Only 3 strains fermenting mannite have been encountered in about 250 hemolytic streptococcus cultures; all were from scarlet fever and none were agglutinated by the immune serum. Another strain which was not agglutinated was isolated from the lochia of a puerperal sepsis patient with clinical scarlet fever. In this case no hemolytic streptococci were isolated from the throat at the onset of the infection. The third culture was isolated from a nurse

with an acute sore throat, a faint general erythema, most marked around the elbows, followed by arthritis and desquamation on the hands and feet. None of these 3 strains absorbed the specific agglutinins from the immune serum.

In two other cases of surgical scarlet fever, hemolytic streptococci isolated from the wound on the forehead and from a burn on the arm, as well as from the throat, were agglutinated by the immune serum.

A hemolytic streptococcus isolated from the finger of a nurse with a sore throat was shown to belong to the scarlatinal group both by agglutination and absorption tests. The throat culture was not so agglutinated.

Two patients admitted with a diagnosis of diphtheria with profuse purulent secretion from the nose, one with enlarged cervical glands, which discharged later, showed no diphtheria bacilli, but almost pure cultures of hemolytic streptococci. Absorption and agglutination tests indicated their scarlatinal origin. A diphtheria patient in the same room contracted a typical attack of scarlet fever except for desquamation 4 days later, which suggested that the patients had had scarlet fever, although there was no such history.

A severe case of scarlet fever in a child, following a cleft palate operation, infected 3 nurses. One had a typical attack of scarlet fever, one a severe sore throat with little eruption, while the third showed only sore throat. The hemolytic streptococci from the patient and the three nurses were all agglutinated in high dilutions by the immune serum. The streptococcus from the nurse without any exanthem absorbed the agglutinins as readily as those from the patient and the nurse with a typical eruption. This case appears to be in the same class as 2 described by Bliss² and 2 by me¹⁵ of hemolytic streptococci specific for scarlet fever from patients exposed to scarlet fever who had acute tonsillitis without exanthem.

The other nonscarlatinal strains were not agglutinated by the immune serum. Absorption tests with 17 indicated also that they did not belong to the scarlatinal group of streptococci.

The absorption experiments corresponded in every case to the agglutination tests.

SUMMARY

Immune sheep serum containing specific opsonins and agglutinins for hemolytic streptococci from scarlet fever retained its specific antibodies for eleven months in the icebox.

¹⁵ Jour. Infect. Dis., 1921, 29, p. 91.

The serums of 2 sheep, specific for hemolytic streptococci from scarlet fever and erysipelas, respectively, produced nonspecific streptococcus opsonins and agglutinins after 8 to 10 months' immunization.

During the course of immunization of a sheep with a hemolytic streptococcus from scarlet fever, some lots of serum became nonspecific on standing in the icebox; some were nonspecific when drawn; some were specific in dilutions above 1:20. Later the serum was specific for streptococci from scarlet fever and remained so on standing in the icebox, but it lost its agglutinating power rapidly.

Rabbits immunized with frequent large doses of hemolytic streptococci from scarlet fever, tonsillitis and sinusitis, produced only nonspecific agglutinins and opsonins. Rabbits immunized with infrequent and somewhat smaller doses of hemolytic streptococci from scarlet fever generally produced specific agglutinins.

Some sheep and rabbits do not produce antibodies in any appreciable amount.

Agglutination and absorption experiments with specific immune rabbit serum confirm previous observations that most of the hemolytic streptococci associated with scarlet fever belong to a distinct biologic group.

EXPERIMENTAL MEASLES BY INOCULATION OF MONKEYS, GUINEA-PIGS AND RABBITS WITH A GREEN-PRODUCING DIPLOCOCCUS

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In earlier papers one of us¹ has described a small gram-positive diplococcus, which was isolated from the blood, eye, nose, throat and sputum of patients with measles, during the preeruptive and early eruptive stage of the disease. The organism was cultivated only in anaerobic cultures from the blood, but it generally grew aerobically in the second generation. The diplococcus could be cultivated readily from the respiratory tract, producing small green colonies on aerobic blood-agar plates. When first isolated and small these cocci generally passed through a tested Berkefeld N filter. Diplococci corresponding morphologically to those in cultures could be demonstrated often in large numbers in smear preparations from the tonsils, nose, sputum and eye.

The blood of patients with measles showed a distinct increase in specific agglutinins and opsonins for this diplococcus from the third to the eighth day following the appearance of the eruption. Complement fixing bodies were present only to a slight extent as the symptoms subsided.

Blood of rabbits, immunized with 4 strains from the blood, eye, ear and nose of patients with measles, showed specific opsonic and agglutinating power not only for the strains used in immunization, but for others from the blood, nose, throat and sputum.

This diplococcus was pathogenic for the rabbit, dog, mouse and rat but not for the guinea-pig, when injected intravenously or intraperitoneally, but no rashes or symptoms of measles were observed in the earlier experiments.

At the height of the attack of measles, gram-positive bacilli and other organisms were sometimes isolated from the blood, but as they were not found in the preeruptive stage, when the blood is known to

Received for publication June 24, 1922.

¹ Tunncliff, R.: Jour. Am. Med. Assn., 1917, 68, p. 1028; Jour. Infect. Dis., 1918, 22, p. 402; Jour. Am. Med. Assn., 1918, 71, p. 104. Tunncliff, R., and Brown, M. W.: Jour. Infect. Dis., 1918, 23, p. 572. Tunncliff, R.: Ibid., 1919, 24, p. 76; p. 181.

be infective, and no opsonins, agglutinins or complement-fixing bodies (unpublished) could be demonstrated for these organisms, they were not considered of any etiologic significance.

The successful intratracheal inoculation of monkeys with measles virus by Blake and Trask,² led to the use of this method, with one exception, in the following experiments: Virus was obtained by washing the nose and throat of patients with measles in the preeruptive or early eruptive stages, with salt solution, or by carefully swabbing the nose and throat and washing the swabs in salt solution, or in a few instances incubating them in ascitic dextrose broth for 24 hours, centrifuging and suspending the sediment in salt solution. The cocci were isolated from the original swabs on aerobic goat blood agar plates. Single colonies were inoculated into ascitic dextrose broth and on blood agar slants. The ascitic dextrose broth cultures were centrifuged and the cocci suspended in salt solution. When the growth on blood agar was used it was washed off in salt solution. The cocci were small, gram-positive, round or slightly elongated diplococci, bile insoluble, fermenting lactose and salicin but not mannite or inulin. They grew in pairs or short chains in broth and produced small green colonies on blood-agar plates.

MONKEYS

Exper. 1.—April, 1918. A rhesus monkey was injected intravenously with the sediment of 100 cc horse serum dextrose broth culture of coccus isolated from blood of a patient with measles 6 hours before the appearance of the eruption. This was the third generation of the organism. The monkey was very ill for one day and slightly so for a few days following the injection. It then remained well for 10 days, when it became less active. On this day the face was flushed, but no eruption was observed either in the mouth or on the skin. There was no rise in temperature. A distinct leukopenia was present from the fourth to the thirteenth day. This reaction was considered due to the coccus because two other monkeys inoculated with equally large numbers of spirilla isolated from the blood of patients with measles produced no such change.

Exper. 2.—March 26, 1921. A monkey was injected intratracheally with 4 cc of salt solution suspension of the growth of 4 colonies of cocci from the nose and throat of a patient with measles with Koplik spots, 24 hours before the appearance of the eruption. When inoculated the face of the monkey was flushed, the eyes were somewhat injected, and bright red macules were present at the outer angles of the eyes. The mucous membrane of the mouth was rather rough and red. The animal remained well and active for 8 days, when it became quiet. On the eleventh day the mucous membrane of the mouth was more congested and on the twelfth day it appeared red and rough with blister-like whitish spots, suggestive of Koplik spots, especially at the angles of the jaw; the mucous membrane of the mouth remained rough and red throughout the experiment. There was diarrhea on the twelfth day. On the following day

² Jour. Exper. Med., 1921, 33, p. 385.

the monkey was listless, the eyes somewhat watery and the face more flushed than usual. The temperature was slightly elevated on the tenth day and remained so most of the time for 10 days, during which period there was a decided fall in the number of leukocytes. Anaerobic blood cultures on the fifteenth day were negative. Cultures from the mouth on the tenth day showed green-producing diplococci which were much smaller than those normally present and which appeared similar to those injected.

This monkey was reinoculated intratracheally with 6 c c of nasopharyngeal washings from a measles patient with Koplik spots, 24 hours before the appearance of the eruption. This injection was followed by no rise in temperature, no exanthem, and no symptoms of measles, except a drop in the number of leukocytes from the fifth to the tenth day following this injection.

Exper. 3.—May 13, 1921. An intratracheal injection was made with a monkey with 8 c c of salt solution suspension of growth of four colonies from throat of patient with Koplik spots, one day before the appearance of measles eruption. When injected there was a marked erythema on the face of the monkey, with a macular eruption around the eyes, which were injected, the inner canthus being swollen and red. Mucous membrane of mouth was bleeding, rough and red. Four days after the injection the monkey was less active and lost its appetite. The mucous membrane on the buccal surfaces were red and rough with whitish spots, suggestive of Koplik spots, but they persisted throughout the experiment. On the fifteenth day the face was more flushed than usual especially around the nose. One bright red papule, 1 mm. in diameter, appeared on the left cheek. It disappeared the following day. On the sixteenth day the face was more flushed, there was slight photophobia and another small bright pink papule appeared on the chin disappearing the following day and leaving some desquamation. On the seventeenth day there were also some copper colored macular patches on the chest with scales. There was a slight elevation of temperature on the sixteenth day, when the leukocytes dropped to their lowest point. There was a leukopenia from the tenth to the nineteenth day. A blood culture made on the sixteenth day was negative. Cultures from the mouth were made several times, but no green-producing diplococci were found which corresponded morphologically to those injected. This animal was later inoculated with the growth of 3 tubes of ascitic dextrose broth cultures of the same culture as used originally, but no rise in temperature, no leukopenia, no eruption and no symptoms were produced.

Exper. 4.—May 13, 1921. An intratracheal injection was made into a monkey with 7 c c of salt solution suspension of growth of cocci from the throat of a child at the onset of the measles eruption. At the time of injection the skin of the face and the mucous membranes of the mouth of the monkey were pale. The animal remained well for 8 days when it appeared quiet. The following day the inner canthus of the eyes was congested. On the tenth day a small discrete bright red spot appeared on the left buccal mucous membrane. Two days later the mucous membrane of the lips was congested. On the fifteenth day there was a slightly red granular patch on the left side of the buccal mucous membrane, which appeared on the right side the following day. This condition disappeared by the twenty-first day. Cultures from these areas showed almost pure cultures of green-producing diplococci, which were small, round or slightly elongated. They disappeared with the congestion. On the eighteenth day a bright pink papule, about 2 mm. in diameter, appeared at the angle of the right eye and a bright pink macule on the left. They disappeared on pressure. The following day another small red papule appeared behind the right ear and a few pinkish macules on the cheek. The next day the eruption had disappeared, except around the eyes where it persisted 3 days,

leaving a few scales. Another bright pink papule appeared on the neck on the twenty-first day. On the seventeenth and eighteenth days there was a slight rise in temperature. There was a marked leukopenia from the ninth to the twenty-first day. Anaerobic blood cultures made on the eighteenth day were negative.

The papule which appeared on the twenty-first day was excised, fixed in Zenker's fluid and stained with alum-hematoxylin and eosin followed by Gram's stain. The sections showed a slight proliferative and exudative reaction about the capillaries and small veins in the upper part of the corium. The endothelial cells of the capillaries were swollen, and there was an accumulation of endothelial leukocytes around them. A few of these cells were in mitosis. A few eosinophils and polymorphonuclear leukocytes and lymphocytes were present around the vessels. Similar changes were seen around the sebaceous glands. In one place there was slight exudation of serum and infiltration of the epidermis with endothelial leukocytes. Some of these cells in the lower part of the epidermis were in mitosis. No necrosis was observed in the epidermis, perhaps due to the age of the papule. The sections were compared with sections of skin from early human measles and corresponded closely. The changes in the corium appeared similar to those described by Ewing³ and Mallory and Medlar⁴ in human measles and by Blake and Trask² in measles in monkeys.

This monkey was reinoculated with 8 cc of washings from the nose and throat of a patient with measles with Koplik spots, at the onset of the rash. Thirteen days later the temperature rose from 101.8 to 103 F. and remained above 102 for 2 days. On the thirteenth day one bright red spot appeared on the left buccal mucous membrane. A few pink macules were observed on the chin and a yellowish maculopapular eruption on the abdomen. On the head, arms and abdomen was an uncolored papular eruption. The animal did not appear sick. The following day a slightly pinkish granular rash was seen on the lower labial mucous membrane and 3 bright red spots on the mucous membrane of the upper lip. The pinkish maculopapular exanthem extended around the mouth and on the thighs. The next day the mouth appeared normal. The eruption had extended to the upper part of the tail but was fading on the face, leaving fine scales. On the seventeenth day the rash was all gone. Desquamation in large flakes was observed on the arms and abdomen. No appreciable leukopenia occurred after this inoculation.

A small green-producing diplococcus was isolated from the mouth on the fourteenth day.

Sections of skin removed on this day showed the same changes seen in the section of skin removed following the injection of diplococci, except that there was a larger number of eosinophils and polymorphonuclear leukocytes around the vessels and one vesicle in the epidermis, infiltrated with polymorphonuclear leukocytes.

These experiments indicated that the diplococcus isolated from the blood and secretions of early cases of measles produced a mild reaction in monkeys; in one case protecting against a subsequent inoculation of virus, in a second against another injection of cocci. The last experiment suggests that the attack was too mild to protect against a possible overwhelming dose of virus. According to Kerr,⁵ relapses in

³ Jour. Infect. Dis., 1909, 6, p. 1.

⁴ Jour. Med. Research, 1920, 41, p. 327.

⁵ Infectious Diseases, p. 52.

cases of measles are exceedingly rare. He has seen only one case in which a typical attack was followed 7 to 8 days later by a repetition of the disease with equally classical symptoms. Osler⁶ says that relapses are occasionally seen, "the symptoms recurring at intervals from 10 to 40 days, but it is not always easy to say whether there may not have been a new infection from without."

GUINEA-PIGS

Ten guinea-pigs were insufflated intratracheally with from 1 to 2 cc of material through a small rubber catheter, as described by Rosenow.⁷ The hair on the chest and abdomen was removed with clippers. Two animals were inoculated with measles virus, 5 with the second and third generations of the green-producing diplococci isolated from this material and 3 with hemolytic streptococci from scarlet fever. One guinea-pig inoculated with virus showed no leukopenia, but a rise of temperature beginning on the seventh day. One injected with diplococci from this material died on the second day with bronchopneumonia. The guinea-pigs inoculated with throat virus and the 4 with measles cocci showed a drop in the number of leukocytes on the fourth and fifth days, coincident with or followed by a rise in temperature of 2 to 3 degrees. This reaction rarely persisted more than one day. An occasional animal coughed, showed some coryza, conjunctivitis and injection of the mucous membrane of the mouth, but Koplik spots and eruptions were observed in none. Green-producing diplococci similar to those injected were isolated from the lung of the guinea-pig with bronchopneumonia and from one with coryza.

Two guinea-pigs injected with hemolytic streptococci showed a decided rise in the number of leukocytes on the fourth and fifth days, without a rise in temperature; the third guinea-pig died on the seventh day with a great reduction in the number of leukocytes and a slight rise in temperature.

These experiments suggest that the virus, as well as the diplococcus from measles, produces in guinea-pigs a leukopenia accompanied by a rise in temperature with no marked respiratory symptoms and no eruptions.

Since these experiments were made Duval and D'Aunoy⁸ have shown that guinea-pigs react specifically to intracardiac injections of defibrinated blood from cases of human measles. They observed a definite and constant rise in temperature and a coincident decrease in the total number of leukocytes after an incubation period of 9 to 12 days. A typical exanthem was not noted. Coryza and injections of the buccal mucous membrane occurred in some animals but were so inconstant as not to be included as part of the specific reactions. An intense hemorrhagic nephritis was a constant pathologic finding.

The difference in the incubation period between their guinea-pigs and ours may be due to the source of the material and method of injection.

RABBITS

Nevin and Bittman⁹ and Grund¹⁰ appear to have demonstrated that rabbits are susceptible to the virus of measles and within rather wide

⁶ Practice of Medicine, Ed. 9, p. 348.

⁷ Jour. Am. Med. Assn., 1919, 72, p. 1608.

⁸ Jour. Exper. Med., 1922, 35, p. 257.

⁹ Jour. Infect. Dis., 1921, 29, p. 429.

¹⁰ Ibid., 1922, 30, p. 86.

limits give a characteristic syndrome. Nevin and Bittman used blood from human patients and Grund the nasopharyngeal secretions from the same patients. They found the temperature and blood counts unreliable. Conjunctivitis and inflammation of the upper respiratory passages in varying degree of severity were noted. They observed eruptions both in the mouth and in the skin after an incubation period of from 2-8 days. Typical Koplik spots were found by Nevin and Bittman in about 33% of the rabbits, in 15% by Grund; eruptions were observed 100% by Nevin and 75% by Grund. The exanthem, according to Nevin, was in no instance typical of measles. It varied from a slight flush over the chest and in the axillary region to general redness extending to the groin. Grund observed either a diffuse punctate erythema and following the erythema, sometimes occurring without it, a maculopapular rash which faded after from 2-4 days, and left pigmentation persisting until desquamation began.

We have injected 9 rabbits intratracheally with measles virus, 22 with the green-producing diplococci from measles and 15 with control organisms. The hair of the chest and abdomen and in some, of the back was removed by shaving or by the use of depilatory. Nine of the control cultures—7 staphylococci, 1 hemolyzing streptococcus and 1 producing large moist green colonies—were isolated from the same plates from which the green-producing diplococci from measles were isolated, with the idea that if the virus was carried by bacteria on the plate it might be transferred as readily by one organism as by another. The other control cocci were 4 strains of *Strep. viridans*, one isolated from a measles patient as the rash was fading, two from the throat of a German measles patient, one from diphtheria, and two strains of hemolyzing streptococci from scarlet fever. All of the organisms injected were of the second generation unless otherwise stated.

Leukocyte counts were made on the first 4 rabbits injected, 2 with virus and 2 with the green-producing diplococci. As no appreciable change in the number of leukocytes was demonstrated, although good rashes were observed, the blood counts were discontinued. A rise in temperature above 104 F. was not constant, occurring in only a few rabbits, but when present it coincided with the appearance of the exanthem. A temperature of 103.8 F. is not infrequent in uninoculated rabbits. Conjunctivitis, coryza and reddening of the buccal mucous membranes were rarely more marked than in the uninoculated rabbits, but small, slightly elevated red spots with whitish centers, which we interpreted as Koplik spots, were observed. The centers were never bluish, but neither were those in the human cases from whom the material was obtained. These spots were seen in 42 per cent. of the rabbits injected with diplococci from measles but were never observed in rabbits inoculated with virus; this may have been due to the smaller number of rabbits so injected. Five spots were seen in 1 rabbit, 2 in another, but as a rule only one would occur on one side and sometimes another on the opposite side, the following day. They appeared in from 3 to 15 days after inoculation, generally about the seventh day, and persisted from 1-3 days. They were followed in from 1-5 days by the eruption which appeared from 7-18 days after injection, generally on the eleventh day. Exanthems

were observed in 87% of the rabbits inoculated with virus, in 84% of those with diplococci from measles. One rabbit with Koplik spots did not show any eruption. The exanthem consisted of a diffuse erythema over the chest, abdomen and back, or of a pink maculopapular eruption of varying extent, with or without erythema, persisting from 1-6 days, generally about 3. The macules were never so red nor so large as in human measles. In some instances the exanthem was followed by pigmentation, generally by desquamation in fine or coarse scales. The redness and desquamation that followed shaving and the use of depilatory disappeared before the animals were injected.

The third, fourth, fifth and seventh generations of the measles diplococci on blood agar still produced Koplik-like spots and eruptions when injected into rabbits. The strain which produced lesions as late as the seventh generation was plated out twice in the interim.

Small pieces of skin were removed during the height of the eruption in three rabbits injected with diplococci from measles. The same changes were found as observed in the lesions in man and monkeys, except that there was a more marked infiltration around the vessels and a larger number of polymorphonuclear leukocytes and eosinophils and more mitoses. The blood vessels showed considerable distention, chiefly with red blood corpuscles, but often with polymorphonuclear leukocytes and eosinophils. There was also greater invasion of the epidermis. The lesions here showed an exudation of serum and endothelial leukocytes into the stratified epithelium, collecting in some places near the outer surface and producing small vesicles. Some of the endothelial cells were multiplying. No bacteria could be demonstrated in the skin sections.

Koplik-like spots were removed from 3 rabbits and found to correspond to the skin lesions except that the invasion of the epithelium was more marked. In one instance an erosion was observed. The microscopic pathology of these spots is similar to that of Koplik spots in man and monkeys.^{2, 3, 4} Gram-positive coccus-like bodies in pairs were seen in 2 specimens outside and inside cells around some of the vessels. They appeared somewhat like the bodies described by Mallory and Medlar in the skin of patients with measles. However, it is very difficult to differentiate cocci from granules in such sections.

As pointed out by Grund, there are a fairly large number of refractory rabbits, only 1 animal out of 2 injected with the same material developing symptoms. In successfully inoculated animals there is a leakage from the nose. In 2 rabbits injected with measles diplococci in which there was no leakage, no symptoms occurred. Although 2 other rabbits receiving the same material reacted, they are included in the series as negative.

None of the rabbits injected with bacteria isolated from the same plate from which the green-producing diplococci from measles were isolated showed Koplik-like spots or eruptions. Four were later reinoculated with virus or measles diplococci and all developed exanthems, 1 Koplik spots. Two rabbits injected with hemolyzing streptococci, 1 from scarlet fever, showed an erythema the following day. Edema of the abdomen was observed in 1 injected with a *Strep. viridans*

from diphtheria. In fact, none of the 15 control rabbits showed Koplik spots or rashes similar to those produced by the virus or diplococci from measles.

Considering that the diplococcus cultures as late as the seventh generation could produce a reaction in rabbits and that they were originally isolated from single colonies and that rabbits similarly inoculated with second generation cultures of staphylococci and green and hemolyzing streptococci from the same plates produced no symptoms, the chance of the selective transfer of the virus by only the green-producing diplococci appears small.

CULTIVATION EXPERIMENTS

The green-producing diplococci are not easily cultivated from the blood of successfully inoculated monkeys and rabbits. In the original work, blood cultures were chiefly made from children acutely ill, into mediums containing ascitic fluid or horse serum. In mild adult human cases they were found difficult to isolate, only two successful cultures being obtained from 20 patients. In one case, the patient had a bronchopneumonia on the day the rash appeared; in the other, the blood was drawn as the exanthem appeared. In both instances the diplococci were isolated in anaerobic Krumwiede¹¹ plain agar plates, without ascitic fluid, as described by Dick and Henry,¹² only 1 or 2 colonies appearing on each plate.

In isolating organisms from the blood of rabbits, plain or dextrose agar plates were made according to the later method of Dick.¹³ The whole blood is added to agar melted and cooled to 42 C. and poured into plates in the usual way. The plates are chilled immediately, and as soon as the agar sets another deep tube of sterile agar, melted and cooled to 42 C., is poured over this surface. The plates are again chilled and the surface covered with a layer of melted paraffin 2-3 mm. thick. The paraffin is sterilized by heating to the smoking point and cooled until a film forms on the surface.

Deep tubes of autoclaved 1% dextrose brain broth, preferably with ascitic fluid, described by Rosenow,¹⁴ were also found useful. Chocolate medium and cultures from the blood, laked with sterile distilled water and centrifuged, were not satisfactory. Saponin to destroy the leuko-

¹¹ *Ibid.*, 1913, 12, p. 199.

¹² *Ibid.*, 1914, 15, p. 85.

¹³ *Ibid.*, 1918, 23, p. 578.

¹⁴ *Arch. Int. Med.*, 1921, 28, p. 274.

cytes as used by Sellards and Bigelow¹⁵ was not tried. On account of their not isolating any diplococci, the conclusion seemed warranted that the saponin might inhibit their growth.

At the last bleeding of an infected rabbit, the citrated blood was centrifuged and cultures made from the top and bottom of the tube, as suggested by Sellards and Bigelow. An abundant growth occurred in ascitic dextrose broth from the top but not from the bottom. No growth was found in cultures of the top fluid when not well diluted, the citrate solution evidently inhibiting growth.

Diplococci similar to those injected were isolated from the Koplik spots and from the blood in 4 of 8 rabbits cultured at the height of the eruption during life and from the lung and blood of 2 other rabbits at necropsy. In 2 cases diplococci were observed in the dextrose brain broth culture, but they would not grow in subculture. One successful culture was taken from a rabbit inoculated with virus, the cocci being isolated from the blood at the height of the eruption on an anaerobic plate and in dextrose brain broth with ascitic fluid. Only one colony appeared on the anaerobic plate, none on the aerobic.

The diplococcus isolated from the lung of the rabbit dying of a bronchopneumonia when injected into another rabbit produced Koplik spots on the third day after injection followed by an eruption on the fifth day.

Two rabbits were injected with the third and fourth generations of the diplococcus isolated during life from the blood of a rabbit inoculated with a diplococcus from the throat of a patient with measles in the pre-eruptive stage. A Koplik spot and eruption was produced in one rabbit on the ninth day and an eruption in a second on the eleventh day. The blood of one of these caused good Koplik spots on the ninth and an eruption on the twelfth day in an inoculated rabbit.

The blood of a rabbit inoculated with a diplococcus was taken at the height of the eruption on the eighth day and inoculated intravenously into 2 rabbits, both of which showed an eruption on the sixth day. The blood of 1 of these was inoculated into another rabbit which developed an exanthem after 3 days' incubation, with a temperature of 105 C. A single colony of green-producing diplococci was isolated from the blood on an anaerobic plain agar plate without ascitic fluid. The experiments indicate that the incubation period is shortened when the organism is passed through animals.

¹⁵ Jour. Med. Research, 1921, 42, p. 241.

REINOCULATION

One rabbit successfully inoculated with virus and 3 with the diplococcus, on reinoculation 6-10 days later with fresh virus, gave no signs of reaction, while the control rabbits showed distinct rashes, 2 a well marked rise in temperature.

FILTRATION EXPERIMENT

As stated, the green-producing diplococci from measles, when first isolated and grown in medium in which they appear minute such as ascitic dextrose broth and dextrose brain broth, as a rule, pass through tested Berkefeld N filters. As most observers have found that apparently sterile filtrates produce the symptoms of measles in monkeys and rabbits, late subcultures which did not appear to pass through the filter and unfiltered cultures of the same strain and generation were injected intratracheally into 4 rabbits. One rabbit injected with the filtrate showed a Koplik spot on the seventh day, followed by an eruption on the twelfth day; the other 3 rabbits developed rashes—2 on the ninth and 1 on the tenth day. The filtered cultures grossly did not appear to grow, but on careful examination diplococci were demonstrated in smears of the original filtered material and slight growth occurred in subcultures on goat blood, dextrose broth and brain broth. In some instances the diplococci grow out slowly in the original filtrate but multiply more readily on subcultures, especially, if anaerobic.

Rosenow¹⁶ has also observed that green-producing streptococci from influenza may pass through filters, through which *B. prodigiosus* will not pass, and that they can multiply and grow when injected into the trachea of guinea-pigs and be recovered from the lung, even though the filtered cultures remain negative. Otlitsky¹⁷ has shown that filtrates of typhus virus which are free, so far as they could ascertain from a living, multiplying agent, can occasionally induce in guinea-pigs not only typical lesions of the disease, but also immunity to later injections of the active virus.

SUMMARY

Monkeys, guinea-pigs and rabbits are susceptible to measles when inoculated with the washings of the nose and throat of patients with measles. The same symptoms and lesions may be produced in these animals by the green-producing diplococci isolated from the blood and respiratory passages of human measles.

¹⁶ Jour. Infect. Dis., 1920, 26, p. 504.

¹⁷ Jour. Exper. Med., 1922, 35, p. 469.

Rabbits successfully inoculated with washings or the diplococcus show no symptoms when reinoculated with fresh virus.

Green-producing diplococci from the blood and lung of rabbits, successfully inoculated with diplococci from measles, produce Koplik spots and exanthems when injected into other rabbits.

Filtrates of the diplococcus cultures generally show abundant growth, but old cultures containing large forms sometimes multiply only after several days' incubation or on subculture into a favorable medium.

While the experiments indicate that the reaction is due to the coccus itself and not to a separate virus carried by it, on account of the same symptoms being produced by cultures as late as the seventh generation and the absence of the reaction in rabbits injected with other bacteria, isolated from the same plate as the green producing diplococci, it is possible that the diplococcus possesses the selective power of carrying the specific virus of measles.

FACTORS INFLUENCING DEVELOPMENT OF METACHROMATIC GRANULES IN THE DIPHTHERIA BACILLUS

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A study was made of some of the outside influences which may affect the production of granules in the diphtheria bacillus. Factors were sought that might hasten the appearance of granules and make an earlier diagnosis possible, and also those which might delay the appearance of the granules, thus interfering with the accuracy of routine examinations.

Types of granules were studied exclusively as most commonly seen in this locality. It is reasonable to suppose that conditions modifying granule formation would also modify the formation of bars in that type of organism.

The appearance of granules at different ages of the organism has been studied by Denny,¹ Albert² and others, who have found that granules appear in cultures from 4 to 8 hours old, and attain their largest average size in cultures from 12 to 15 hours old.

The effect of reaction of the medium on granule formation was studied by Layborne² and Bunker.³ Layborne found that blood serum of P_H 7 to 7.5 gave the largest percentage of granule forms. As the reaction approached P_H 5.5 and 8.5 the organism became smaller and the percentage of granule forms less.

Denny⁴ says that the transition from the solid to the granular form depends on either the accumulation of bacilli or products of growth of the organism. Denny also studied the effects of various incubation temperatures in reference to granule formation and found that the appearance of granules was delayed at a temperature of 19 to 21 C., and at a temperature of 40. He also found that symbiotic growth with various organisms delayed the granule production.

Heineman⁵ and Mellon⁶ varied the morphology of the organism, producing a coccus-like form from a granule form by growing it on veal glucose broth and bringing it back to the granule form on blood serum or blood agar.

Wherry⁷ studied the morphology of one culture in relation to oxygen tension. He found that the barred and granule forms disappeared when grown on Loeffler's blood serum under anaerobic conditions and also that growth with *B. subtilis* produced the same effect.

Received for publication July 5, 1922.

¹ Jour. Med. Research, 1903, 9, p. 117.

² Absts. Bacteriol., 1921, 5, p. 14.

³ Ibid., 1917, 1, p. 31.

⁴ Public Health, 1902, 28, p. 471.

⁵ Jour. Bacteriol., 1917, 2, p. 361.

⁶ Ibid., p. 81.

⁷ Jour. Infect. Dis., 1917, 21, p. 47.

Browning⁸ states that the appearance of granules is due to either the method of fixation of the smear or the wash water used in making the preparation. This theory did not need to be considered since granules in this general group can be stained while the organisms are living.

Meador⁹ states that the type may vary because of acid production from glucose.

Arloing and Richard¹⁰ used various mediums, coagulated guinea-pig serum, Costa lecithin medium with beer yeast and phosphorus compounds and found that granules were produced.

SOURCE OF CULTURES

The cultures used were selected from a list of eleven. Nine of these were isolated from routine throat cultures obtained from the diagnosis division of the Municipal Laboratory. One was supposed to be Park 8 and was obtained from Lakeside Hospital. The remaining culture was from a case of clinical diphtheria at Mt. Sinai Hospital. Of these cultures, two were from single cell isolations. All cultures were virulent. These cultures were all good granule forms with the exception of Park 8, which was found to be variable in its granule production and was not used in the later experiments.

TECHNIC

All work was as carefully controlled as possible. On plates, when it could be done, one half of the plate was used as the control and the other half for the experimental work. In making preparations for morphologic study care was taken to obtain portions from as near the same part of the culture as possible.

The staining used was that of Albert,¹¹ except in the earlier work in which his first formula was used. It was the impression that Albert's stain was much more satisfactory for granule demonstration than Loeffler's methylene blue or some of its various modifications which were tried. To exclude possible errors in staining technic the controls were stained on the same slide and at the same time as the organism in the experiment. In most cases a count of 100 cells was made and the cells classified as: (1) granules; (2) unevenly stained cells, including organisms in which part of the cell showed a degree of staining different from the remainder but no definite granule formation; and (3) solid forms. When an examination of a preparation showed that the organisms were practically all of one type, a count

⁸ Applied Bacteriology, 1918, p. 58.

⁹ Jour. Infect. Dis., 1919, 24, p. 145.

¹⁰ Bull. de l'Inst. Pasteur, 1921, 19, p. 738.

¹¹ Jour. Am. Med. Assn., 1921, 76, p. 240.

was not made, and the general impression of the type was given. Detailed tabulations of the results of counting are not given, and in the three tables the results of counting a number of cultures have been averaged in order to show in a condensed form the approximate granule, unevenly stained and solid forms.

PRESENCE OR ABSENCE OF CERTAIN NUTRITIVE MATERIAL IN THE MEDIUM

The incubation time was about 16 hours in all cases. A serum agar medium made after the manner of Klein,¹² but with ox blood serum in place of horse serum, was used extensively. The number of granule forms and the number of granules in each organism on this medium are about the same as on Loeffler's blood serum, although the organisms themselves are for the most part smaller in size. As Loeffler's blood serum contains dextrose while the Klein medium does not, 1% dextrose was added to Klein medium. The number of granule forms and the size of the organisms were the same as the Klein medium without dextrose.

Other mediums which gave a percentage of granule forms almost equal to that obtained on Klein medium were: blood serum digested with trypsin until it would not coagulate on boiling and then added to agar; Frost medium;¹³ ordinary laboratory milk medium; serum water; a mixture of equal parts of milk and serum water; slants of egg yolks and egg white; Whittaker's¹⁴ casein agar.

Mediums which gave a morphologic form and granule production similar to that seen on glycerol agar were Ayers and Mudge¹⁵ milk powder medium; Huntoon's¹⁶ hormone medium; Hitchens' lower percentage agar medium¹⁷ and potato slants. Eberson's yeast medium¹⁸ produced a peculiar coccus-like form with no granules. Gelatin produced few granule forms. Four cultures were grown on the following mediums with indicated results; the counts in each case are averaged:

Agar + peptone + meat extract = granule forms, 66; unevenly stained, 6; solid, 26.
 Agar + peptone = granule forms, 8; unevenly stained, 12; solid, 79.
 Agar + meat extract = granule forms, 31; unevenly stained, 19; solid, 50.

¹² Deutsch. med. Wchnschr., 1920, 1, p. 297.

¹³ Jour. Am. Med. Assn., 1921, 76, p. 13.

¹⁴ Jour. Am. Med. Assn., 1912, 37, p. 82.

¹⁵ Jour. Bacteriol., 1920, 5, p. 565.

¹⁶ Jour. Infect. Dis., 1918, 23, p. 169.

¹⁷ Ibid., 1921, 29, p. 390.

¹⁸ Jour. Am. Med. Assn., 1919, 72, p. 852.

Comment.—Agar with peptone gave in all four cultures a peculiar short almost ovoid organism. This was thought at first to be a contamination, but transplants on other mediums showed pure cultures to be present. A much higher percentage of granule forms is seen in the medium with meat extract than with peptone alone, but in neither case is the percentage as high as where both were used.

From these experiments it can be seen that:

More granule forms are seen when the basis of the medium is a complex protein nature such as blood serum in its various forms or that of egg or milk protein.

The protein derivatives found in meat extract give better results than those found in peptone when added to a plain agar base.

AMOUNT OF WATER IN MEDIUM AND GRANULE FORMATION

Difco desiccated blood serum was used in these experiments and when made as directed contained about 92% water. Eight cultures were inoculated on mediums made in this manner as controls. The same 8 cultures were then inoculated on the blood serum mediums made with varying amounts of water. The incubation time in all cases was 16 hours at 37 degrees.

The results obtained are given in table 1. In each case figures represent the average of the count of granules, unevenly stained and solid forms of the 8 cultures used.

A marked diminution in the number of organisms showing granules occurred in the mediums containing 80% and 70% water compared to the control containing 92% water.

The size and shape of the organisms and the number of granules per organism in the 80% of water were about the same as in the controls. In the 70% water mediums the organisms were slightly shorter. The results with mediums of less than 60% were inconclusive as the granule forms present were practically all of the normal blood serum morphology which were thought to be the remains of the mature granule forms of the transplant.

In view of this possibility, 6-hour growths of the same cultures on ordinary blood serum were inoculated on mediums of 50%, 40%, 30% and 20% water and incubated for 16 hours. The 6-hour cultures were used as controls.

The results are shown in table 2. As would be expected, there were few granule forms in the 6-hour cultures. When these were transferred to the mediums of small water content there was evidence

that some division had taken place in the increased number of young forms. Transplants from these cultures showed growth in several instances.

INFLUENCE OF METABOLIC PRODUCTS OF THE ORGANISM ON GRANULE FORMATION

As Denny had made the statement that granule formation depends on either the accumulation of the organisms or the products of the growth of the organism, this relation to crowding was studied in some detail.

Granule formation of organisms from single and confluent colonies was observed. Eight cultures were streaked on blood serum

TABLE 1
AMOUNT OF WATER IN THE MEDIUM AND GRANULE FORMATION FROM 24-HOUR CULTURES

	92% Water	80% Water	70% Water	60% Water	50% Water	40% Water	30% Water	20% Water
Granules.....	71	53	41	27	13	20	24	22
Unevenly stained.....	10	12	12	18	12	19	10	7
Solid.....	18	35	47	53	75	61	65	70

TABLE 2
AMOUNT OF WATER IN THE MEDIUM AND GRANULE FORMATION FROM 6-HOUR CULTURES *

	92% Water	50% Water	40% Water	30% Water	20% Water
Granules.....	14	5	6	3	5
Unevenly stained.....	23	11	9	6	10
Solid.....	63	83	84	90	85

* Count of 6-hour growth on blood serum with 92% water. The other counts are from 16-hour growths.

plates, Klein medium plates and glycerol agar plates so that there was a heavy growth on one portion of the plate and isolated colonies on another. Plates were incubated for 16 hours at 37 degrees. Preparations were made from single and confluent colonies and counts made and averaged in each case (table 3).

In each medium the granule formation was much more marked in the confluent than in the single colonies.

The question whether the growth products are able to pass through a thin film of agar and influence granule production was investigated. Glycerol agar plates with mediums less than 2 mm. in thickness were heavily inoculated with cultures and incubated for 24 hours. With a sterile razor blade, sections were lifted from these plates and inverted

into sterile Petri dishes. The uninoculated side of each was tested for sterility, and cultures streaked on the sections. Controls were made on glycerol agar plates. The plates were incubated for 8 and 16 hour periods. The counts tabulated in table 3 are the average of 8 cultures in each case.

The results show that in the 8-hour cultures there was a marked increase in the number of granule forms on the inverted sections when compared to the controls. On all inverted sections the predominant type was a short rod with 1 and occasionally 2 granules, the type usually expected in a 16-hour agar growth. The predominant type in the controls was the solid Hofmann or short rod which would be expected. In the 16-hour growths the number of granule forms on the inverted sections was practically the same as on the control medium and also about the same as the number found on the inverted section after 8 hours' observation.

TABLE 3
GRANULE FORMATION IN SINGLE AND CONFLUENT COLONIES METABOLIC PRODUCTS PASSING THROUGH THIN FILM OF AGAR

	Blood Serum		Klein		Glycerol Agar		8 Hours Inverted Sections of Agar		16 Hours Inverted Sections of Agar	
	Sin- gle	Con- fluent	Sin- gle	Con- fluent	Sin- gle	Con- fluent	Con- trol	Inoculated Section	Con- trol	Inoculated Section
Granules.....	17	78	2	86	6	69	15	67	66	53
Unevenly stained..	17	9	9	8	14	14	12	9	7	11
Solid.....	65	13	88	5	79	17	72	24	27	36

The metabolic products of the organisms can thus apparently pass through a thin film of agar and hasten granule production. This effect, however, is seen only in the young (8-hour) cultures. In the older cultures (16-hour) it is not seen, possibly because by this time the inoculated organisms have produced a sufficient quantity of these products so that there is present the maximum number of granule forms possible under the conditions of growth.

The products of organisms killed by moderate heat were utilized for effect on granule formation. Slant agar growths of 8 cultures were killed by moderate heat and then incubated for 3 weeks to allow autolysis to take place. Preparations from these cultures at this time showed organisms of typical agar morphology. Subcultures gave no growth so it was evident that little or no autolysis took place, and this method of obtaining products of the organism had to be abandoned.

Broth filtrates of several organisms obtained after 3, 10 and 13 days' growth were painted on one side of a divided glycerol agar plate after both sides of the plate had been inoculated with the homologous organism and other strains. Plates of glycerol agar, Klein medium and blood serum were used. The untreated side of the plate was used as the control. Eight and 16-hour observations were made, but in no case was there any effect of the filtrate on granule production.

A 24-hour growth of one broth culture was filtered and painted on glycerol agar plates in like manner. The results in this case were doubtful. The organisms from which the filtrate was made showed no increase in granule forms when bathed in the filtrate. Six of the 7 other cultures used did show a slight increase in granule forms in an 8-hour period.

A filtrate heated to destroy toxin, a dilute Schick toxin and a toxin-antitoxin used in the same manner showed no effect on granule formation.

The reaction of the broth was little changed by the growth of the organisms. The broth used had a reaction of P_H 7.4. The reaction of the 24-hour filtrate was between 7.4 and 7.5, and that of the 13-day filtrate was 7.7. Due to this slight change it is improbable that the reaction of the growing culture has any marked influence on granule production.

INFLUENCE OF TEMPERATURE AND GRANULE FORMATION

Eight cultures were used on glycerol agar and Klein medium. These were incubated for 16 hours at various temperatures. The results obtained by counting the cells and averaging the results may be summarized:

Glycerol agar cultures at 27 C. showed a reduction to 56% granule forms from the 73% produced at a temperature of 37 C. At a temperature of 18.5 C. the percentage of granule forms was 20%, and these were for the most part of the morphology of the cultures on Klein medium from which the transplants were made.

Klein medium slants at 37 C. showed 70% granule forms to be present, and at 21 C. there were 27% granule forms. Denny states that a temperature of 40 C. delays granule formation, so a higher than body temperature observation was not used.

OXYGEN TENSION

Eight cultures were grown on 1% dextrose glycerol agar and on Klein medium as controls. In tubes of similar cultures the upper half

was heated for 10-20 seconds and quickly corked with rubber stoppers. The cultures were incubated for 18 hours at 37 degrees.

The results obtained by counting the cells showed that this method of reducing oxygen did not influence granule formation.

Eight cultures grown anaerobically on the same medium showed a slight diminution in the number of granule forms in the dextrose agar and a marked diminution in Klein medium. In the latter medium there was an average of 75% of granule forms in the control and an average of 8% under anaerobic conditions. In the dextrose agar under anaerobic conditions the typical organisms were slightly elongated and coccus-like instead of the short rods containing one or two granules of the control. In the Klein medium the organisms were shorter than in the control but did not show this coccus-like form.

The 8 cultures, when grown at room temperature (18 C.) for 16 hours, under a vacuum which varied from 10 to 19 inches of mercury, showed no reduction in number of granule forms.

SUMMARY

Granules in the diphtheria bacillus are produced in a large percentage of organisms when the culture is grown on a medium of which the base is blood serum. The granules are produced in lesser numbers when grown on glycerol agar. The meat extract in glycerol agar apparently favors the production of granules more than does the peptone. But more granules are produced when both are used. A semiliquid nutrient agar medium does not favor granule formation.

The full number of granule forms develop in a blood serum medium containing 92% of water. When the amount of water is decreased below this amount there is an appreciable decrease of granule forms.

There is a marked decrease in the proportion of granule forms to others when smears are made from a single colony, as compared with smears from confluent colonies.

Products of growth of the organism can apparently pass through a thin film of agar and hasten the granule forms in young cultures. This does not hold true in older cultures.

A broth filtrate of an organism probably does not influence granule production either in that organism or other organisms. Neither diphtheria toxin, heated filtrate nor toxin-antitoxin influence granule formation.

Granule production is better at a temperature of 37 C. than at lower temperatures. Unless the temperature fell below 27 C. the effect was small.

Reduced oxygen tension does not influence granule production unless sufficient to absorb oxygen appreciably from the culture, then few granule forms are produced.

CONCLUSIONS

Denny's observation that the granule appearance depends on either the accumulation of bacilli or the formation of products of the bacilli is confirmed in these observations. The fact that there was an apparent stimulus when it was possible for the products to pass aseptically through a film of medium is most suggestive of the presence of metabolic products, but the lack of success with filtrates from fluid mediums indicates need of more work along this line. The observation on increased granule formation in crowded cultures is suggestive of the same point.

To produce granules in maximum quantity and at maximum speed, diphtheria organisms require suitable mediums, a large amount of water, a growth temperature close to 37 C., a certain amount of oxygen, and the accumulation of large numbers of organisms.

EXPERIMENTAL BOTULISM IN DOGS

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As a part of an experimental project to determine the effect of botulinus toxins on different animals, dogs have been fed and injected with unfiltered A and B toxins of *Clostridium botulinum*. The effect on dogs of ingesting the uncooked animal carcass dead of artificially induced botulism was also noted. In a review of the available literature, the susceptibility of dogs to botulinus toxins is not found definitely stated, though Kempner¹ reported that a cat developed profound symptoms of intoxication following the feeding of toxin at intervals. The symptoms observed consisted of local paresis of the hind quarters, slight dilatation of the pupils, hoarseness, and general emaciation. Dickson² also employed cats in microscopic studies of botulinus intoxication.

The toxins we used were produced by seeding the spores of *Clostridium botulinum* in glucose pork infusion broth. After an incubation period of 6 or more weeks at room temperature the toxicity was determined on guinea-pigs. Unfiltered toxins given subcutaneously to guinea-pigs (250 gm.) in 0.001 c c doses produced death in less than 24 hours. Some of the toxins were doubtless lethal to guinea-pigs in smaller amounts, but the minimum lethal dose was not determined. All toxins used in the dog experiments were unfiltered and unless otherwise stated contained the spores and a few vegetative forms of the organism.

The toxicity of the carcasses fed to dogs from cases of artificially induced botulism was not determined, yet from the tissues of some of the animals taken immediately following death, the spores were occasionally demonstrated by cultural methods. In similar fatal cases (pigs) toxin has at different times been demonstrated in the blood stream following death.

SUSCEPTIBILITY OF DOGS TO TYPE A TOXIN

Type A toxin was given to 18 dogs subcutaneously in amounts varying from 0.001 c c to 10 c c (table 1). The course and severity

Received for publication July 6, 1922.

¹ Ztschr. f. Hyg. u. Infektionskr., 1897, 26, p. 481.

² The Rockefeller Institute of Medical Research, Monograph No. 8, 1918.

of the illness produced varied in different animals in direct proportion to the amount of toxin given and the weight of the dog. Manifest symptoms were observed as early as 12 hours after exposure, though in most cases 36 to 48 hours elapsed before symptoms were noted. Two dogs that received 0.1 c c subcutaneously died after protracted illness, and 7 dogs that also received 0.1 c c subcutaneously showed symptoms but gradually recovered. A smaller amount (0.001 c c) of toxin failed to produce illness in one animal while 1 c c subcutaneously produced death in 3 of the dogs. Antitoxin apparently protected one of the dogs that received 1 c c of toxin.

TABLE 1

TYPE A TOXIN OF CLOSTRIDIUM BOTULINUM ADMINISTERED SUBCUTANEOUSLY TO DOGS

Date	Weight, Lbs.	Dose, C c	Antitoxin	Results
9/26/21	15	0.001	Remained healthy
9/26/21	15	0.01	Remained healthy
9/26/21	14	0.1	Symptoms of botulism developed in 18 hours; died 9/28/21
3/ 2/22	19	0.1	3/4, stiffness; 3/6, prostration and inanition; 4/10, chronic condition
3/22/22	30	0.1	3/25, slight stiffness and dulness; 3/27, recovery noted; 4/3, recovery complete
3/22/22	31	0.1	Remained healthy
3/22/22	23	0.1	3/25, slight stiffness; 3/29, stiffness aggravated; 4/10, recovery almost complete
3/22/22	25	0.1	3/24, stiffness and incoordination; 3/27, decumbent; 4/10, recovery complete
3/22/22	28	0.1	500 units subcut. 20 days previous	Remained healthy
3/22/22	18	0.1	3/24, stiffness; 3/25, decumbent; 4/1, died
3/22/22	31	0.1	3/24, slight stiffness and incoordination; 3/27, decumbent, emaciated and inanition; 4/10, chronic condition
9/16/21	8	1	Died after 12 hours
9/16/21	14	1	Died after 12 hours
9/16/21	7½	1	500 units subcutaneously	Remained healthy
1/23/22	25	1	Died 1/25/22
9/16/21	10	No treatment	Remained healthy
11/ 8/21	..	10	Sudden illness; died 11/10/21
11/ 8/21	..	10	Sudden illness; died 11/10/21

One c c of unfiltered toxin contained more than 1,000 lethal doses for a 250 gm. guinea-pig.

Type A toxin of *Clostridium botulinum* fed daily for 30 days to a puppy (6 weeks' old) in amounts of 1 c c in milk failed to induce ill effects. Doses of 10 c c to 60 c c of type A toxin administered with pipet or drinking bottle to dogs weighing 10 or more pounds which had been receiving normal rations up to the time of treatment, failed to cause noticeable ill effects. In attempting to determine the effect of acid gastric juice on toxin, the stomach contents of 2 dogs were alkalinized by feeding 50 c c of a 5% sodium bicarbonate solution

followed by 50 c c of type A toxin. In neither case were any noticeable effects observed.

Seven dogs from which food had been withheld for 48 hours before being given 30 c c to 60 c c of type A toxin by mouth developed noticeable symptoms of botulism in 2 to 4 days, followed by spontaneous recovery. A dose of 100 c c produced more profound symptoms terminating in death in some cases about the sixth day.

Dogs receiving 5 c c or more of type A toxin subcutaneously suddenly developed symptoms of profound weakness after an interval of 12 to 36 hours. The affected animals were unable to stand or move but remained in a decumbent position. The muscles were flaccid. Their mental condition remained normal for several hours after the development of the weakness as evidenced by the expression of the eyes and the wagging of the tail when spoken to. Animals in this condition frequently died within 24 hours after the onset of the symptoms. All died within 48 hours.

Dogs receiving smaller doses of type A toxin subcutaneously or sufficiently large doses by mouth developed symptoms more slowly. Affected animals walked stiffly with short steps, followed by incoordination of movement and swaying from side to side. Later, when attempting to walk, they would fall forward on the head. Exertion in regaining the feet was sometimes followed by jerky incoordination of muscles and trismus. In 10 to 24 hours animals in this condition were completely unable to stand. Lacrymation and salivation often accompanied these symptoms. Affected dogs seldom barked, but when forced to move emitted a hoarse, suppressed bark or whine. They refused food and water. Animals decumbent for 2 or 3 days frequently suffered from a swollen tongue which partially protruded from the mouth. The saliva was not excessive but dripped slowly from the lips. At first the saliva was clear and without odor, but later it became a brownish color and fetid in character. A severe cheilitis developed in one case. Respiratory distress was observed in the latter stages of the disease in some dogs.

ANATOMIC CHANGES

Dogs which had received fatal amounts of type A toxin showed a general passive congestion of the mucous membranes, internal organs, brains and meninges. Dogs that survived 3 or 4 days had little solid ingesta in the stomach and small intestine. The content consisted of mucous secretion. The posterior bowel contained firm and more or

less dry feces. Dogs that lingered for a greater length of time showed severe bile imbibition throughout the digestive tract.

SUSCEPTIBILITY TO TYPE A SPORES

Spores of *Clostridium botulinum* type A prepared by washing 5 or more times and centrifugalizing were detoxicated by heating at 80 C. for 15 minutes. One c c (3,800,000 spores per c c) suspension injected subcutaneously into guinea-pigs failed to induce ill effects. The viability of the spores suspension was established by seeding into glucose agar. Doses varying from 5 c c (19,000,000 spores) to 25 c c (95,000,000 spores) were injected subcutaneously into dogs. No ill effects were observed.

SUSCEPTIBILITY TO TYPE B TOXIN

Fifteen dogs, varying in age and size from nursing pups to old dogs weighing 30 pounds, were given *Clostridium botulinum* toxin, type B, subcutaneously, in doses varying from 1 c c to 80 c c. Soreness at the point of injection was generally noticed for the first 24 hours, but no other ill effects could be observed. Dogs kept for several months after the injection of 10 c c of toxin containing spores showed no tendency to develop the chronic type of the disease. Doses of 5 c c and 15 c c of type B toxin were injected intravenously into two dogs. A few hours after injection, slight transitory dulness was noted, followed by complete recovery.

THE EFFECT OF FEEDING CARCASSES OF ANIMALS THAT DIED OF BOTULISM

Fifteen dogs were fed ad libitum, the muscle, liver, kidney, heart, and lung tissues of 2 horses and 6 pigs that had died of experimentally induced type A botulism. The material was fed before spoilage could occur and was consumed in liberal amounts by the dogs. No noticeable ill effects were observed in the dogs ingesting this material.

SUMMARY

Unfiltered type A toxin of *Clostridium botulinum* proved fatal to dogs in doses of 0.1 c c or more administered subcutaneously. Type A spores, detoxicated by washing and heating, produced no noticeable effects when injected subcutaneously into dogs.

Feeding relatively large amounts of unfiltered type A toxin (100 c c) produced illness and death in dogs only when food had been previously

withheld for 48 hours. The effects of other forms of artificially induced fatigue were not determined.

The symptoms of type A intoxication included loss of appetite, muscular weakness, languor, prostration, salivation, congestion of the mucous membranes of the mouth, and respiratory disturbances.

Type A antitoxin apparently protected dogs against lethal amounts of toxin.

Unfiltered type B toxin of *Clostridium botulinum* administered intravenously and subcutaneously in liberal amounts failed to induce manifest symptoms.

• The internal organ and muscle tissues of horses and pigs which had died of type A botulism were consumed by dogs without ill effects.

Dogs appear to be satisfactory animals for differentiating A and B toxins of *Clostridium botulinum* following subcutaneous injection

FACTORS GOVERNING THE FAT CONTENT OF BACTERIA AND THE INFLUENCE OF FAT ON PELLICLE FORMATION *

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This paper deals with the acetone, petrol ether and ether extracts of various organisms grown with different substances as sources of carbon.

The study of the fat content of plant and animal cells is a most difficult problem. In the case of bacterial cells this is particularly true; first, because of the difficulty in obtaining large quantities with which to work; and second, because of difficulties in extracting the pure fats from any medium which contains water. The latter becomes a serious obstacle in the fat extraction of bacteria because there is no satisfactory method of drying the organisms without changing the structure and chemical constituents of the cell. For this reason this work was conducted by using acetone which would serve both as a dehydrant and a fat extractive and would remove the obvious objections to dehydrating the organisms by means of heat, sulphuric acid, alcohol, etc. The results obtained cannot be proved to represent an absolute criterion of the fat content for each organism since acetone and ether will extract substances other than fats, but in each case the results are comparable to those of every other case, because of the uniformity of medium, the time allowed for growth of the organisms, and the similar method of extraction.

One of the greatest obstacles confronting earlier workers on the analysis of bacterial cells was the difficulty in obtaining large enough quantities of the organisms with which to work. Centrifuges suitable for this type of work were almost unused up to the last decade, and the primitive and laborious method of scraping the growth from agar plates or skimming it from liquid mediums prevailed. For this reason the work on bacterial cell analysis is comparatively meager and restricted to certain types of bacteria. The tubercle bacillus has been most extensively studied probably because of its abundant growth on glycerol broth.

Received for publication, July 7, 1922.

* Aided by a Grant from the National Dental Research Association.

Cramer,¹ in 1893, published his results on the composition of bacteria with respect to the character of the medium. He analyzed several organisms for carbon, oxygen, nitrogen, hydrogen, ash and fat content. His method of fat extraction was by means of the Soxhlet apparatus using alcohol and ether as solvents. This method necessarily gave extractives other than fats as alcohol dissolves out other substances. However, his results are of interest. He analyzed the *Bacillus mucous capsulatus* and *Bacillus rhinoscleroma* grown on 1% and 5% peptone with 5% dextrose. His results show about 10% fat for both organisms when grown on 1% and 5% peptone, and 20% (an increase of 100%) when grown on 5% dextrose. He concludes that on easily assimilable medium the composition of the bodies of the same germ from different sources and which vary in virulence is about the same. Where, however, the medium supplies food that is assimilable with difficulty the composition of the bodies of the germs will vary. The comparison which he makes between these and several other species shows a variation which indicates a distinct and characteristic composition for each germ. He does not consider the action of the organism on the medium.

The preliminary work by Hammerschlag² on the substances contained in the bodies of the tubercle bacillus which could be extracted with ether and alcohol, and the probable composition of the extract so obtained, furnished considerable material for speculation. He grew the tubercle bacillus on glycerol bouillon and on glycerol peptone agar and obtained the organisms by skimming the growth from the liquid medium or scrapping it from the agar. He then extracted the organisms with ether and alcohol and dried to constant weights. His results show 28 and 26% fat, respectively, from 2 different strains.

De Schweinitz and Dorset³ reported their results on the fat content of the tubercle and glanders bacilli. They grew large quantities of the organisms on glycerol beef broth and also on ordinary beef broth medium, freed the organisms by filtration and washed out all soluble products with water. The organisms were then dried over sulphuric acid and just before analysis were further dried by heating to 100 C. They state that by means of this drying process the organisms underwent no change in color. They then analyzed the dried organisms for nitrogen, carbon, oxygen, phosphorus, sulphur, ash, etc., and conclude that "with the exception of the nitrogen, there seems to be very little variation in the composition of the germs grown on beef broth or on ordinary media." Their article does not make direct mention of the fat content or just what procedures were followed. Their results show a total ether extract of the tubercle bacillus grown on beef broth averaging 39.29% to 40.8% but no alcoholic extract; on the artificial medium the ether extract was 37.57% to 38.54% and the alcoholic extract 4.44%.

Subsequent work by Klebs,⁴ Weyl,⁵ Ruppel,⁶ Aronson,⁷ and Levene⁸ substantiated De Schweinitz and Dorset's conclusions that the tubercle bacillus contains from 27 to 38% fat, depending on the strain.

In 1902 De Schweinitz and Dorset⁹ reported further investigations on the tubercle bacillus. They grew the organisms on 7% glycerolated acid potassium

¹ Arch. f. Hyg., 1893, 16, p. 151.

² Centralbl. f. klin. Med., 1891, 12, p. 9.

³ Jour. Am. Chem. Soc., 1895, 17, p. 605.

⁴ Centralbl. f. Bakteriöl., 1896, 20, p. 488.

⁵ Deutsch. med. Wchnschr., 1891, 17, p. 256.

⁶ Ztschr. f. phys. Chemie, 1899, 26, p. 218.

⁷ Berl. klin. Wchnschr., 1898, 35, p. 484.

⁸ Jour. Med. Research, 1901, 6, p. 135.

⁹ Centralbl. f. Bakteriöl., 1902, 32, p. 186.

phosphate broth, washed them with hot water to remove the phosphates, then dried over sulphuric acid, powdered and dried to constant weight at 60 C. in vacuo. Extractions were then made with hot ether, hot alcohol and hot chloroform. They obtained 26.32% total extracts from the bovine strain, 20.59% from the swine strain, 31.76% from the horse strain, 30.65% from the avian strain, 37.41% from the attenuated human strain and 28.03% from the virulent human strain. They assume that the virulent human, horse, bovine and avian strains produce less harmful fatty substances, and therefore a relatively greater amount of poisonous proteid material than the attenuated human strain, and that this might explain the differences in virulence. This is in disagreement with the work of Frouin,¹⁰ who found the reverse to be the case. As will be pointed out later, they do not consider the action of the various strains on the glycerol contained in the medium.

Lyons¹¹ investigated the fat content of 3 organisms. We are unable to determine what micro-organisms he was working with, but it is obvious that the Pfeiffer bacillus he refers to is not the influenza bacillus as we know it today, as his organism was encapsulated and grew well on the routine mediums. Another he designated as number 28 and a third as a threadlike bacillus. He grew these organisms on 1, 5 and 10% dextrose agar, respectively, scraped the growth off, dried in a desiccator and ground to a fine powder. He then obtained ether and alcohol extracts by means of a Soxhlet apparatus. He found that the alcohol extract increased as the amount of sugar in the medium was increased but that the ether extract increased only up to 5% sugar and decreased on addition of more sugar. The sum of the ether and alcohol extracts, however, increased with an increase of sugar in the medium. His method of using alcohol as a solvent is open to criticism for alcohol will dissolve out things other than fats. Hence we must conclude that his total alcohol and ether extracts represent an amount in excess of the exact amount of fat present in the organisms. Since he does not give the action of the organisms on sugar, and since his organisms are not well known today, his work does not contribute much to our knowledge of the fat content of bacteria.

Dawson¹² studied the fat content of the colon bacillus grown on 8 different mediums. He treated weighed quantities of wet bacteria with 20% antiformin for 24 hours at 30 C., then heated to 60 C. for one hour. After cooling to 15 C. and neutralizing with N/10 sulphuric acid, the solution was evaporated to dryness at 80 C., ground and extracted with ethyl ether or petrol ether. The residue was slowly boiled in 10% sodium hydroxide and after boiling was rendered acid with dilute sulphuric acid. It was then diluted to 3 times the original volume with distilled water and cooled to 7 to 10 C. after which it was filtered. The precipitate was washed, dried and extracted with petrol ether for 5 hours. He obtained 3.99% fat when the organism was grown on 1% peptone plus 1% meat extract broth, 5.77% on glucose broth, and 8% on 1% glycerol glucose broth. These figures are comparable with ours, being somewhat smaller. No mention is made of the relation of the medium and the action of the organism on the medium to the fat content.

Froin's¹⁰ work on the fat content of the tubercle bacillus is of great interest. He found that the bovine (with one exception) and the avian strains had high fat contents, ranging from 42 to 54% when grown on glycerol medium while the human strains have about one-half (19 to 21%) as much fat as the bovine and avian strains. He adds that the effect of the glycerol is noteworthy, and

¹⁰ Compt. rend. Soc. de biol., 1921, 84, p. 606.

¹¹ Arch. f. Hyg., 1897, 28, p. 30.

¹² Jour. Bacteriol., 1919, 4, p. 133.

the comparison of the fat and wax content in human and bovine bacilli grown under identical conditions suggests that this chemical factor may be associated with the virulence of the strains for laboratory animals.

The work here reported was inaugurated for the purpose of studying the relation of the fat content of bacteria to pellicle formation. Larson, Cantwell, and Hartzell¹³ have shown that pellicle formation may be suppressed by lowering the surface tension of the culture medium. Larson¹⁴ ascribes this phenomenon to a condition of more complete wetting of the organism by the fluid. It is obvious that factors other than the surface tension of the medium enter into this phenomenon since not all bacteria form pellicles when grown on a medium of given surface tension.

On glycerol broth many organisms will grow with definite pellicle formation even though the surface tension of the glycerol broth is approximately that of ordinary broth. If pellicle formation is an indication of incomplete wetting of the bacteria, it follows that there must be differences in the ability of various bacteria to become wetted.

It seemed to us that differences in the amount and possibly the disposition of the fats of the cell might account for the difference in behavior of bacteria grown under identical conditions. The effect of glycerol and various carbohydrates on the fat content have been studied.

The technic used in this work was as follows: Pure strains of an organism were grown on plain broth, 0.5% dextrose broth and 3% glycerol broth, respectively. The broth contained 12 gm. of Liebig's beef extract, 20 gm. peptone and 10 gm. of sodium chloride to a liter of distilled water, and titrated to 1% acidity. The dextrose and glycerol broth were composed of plain broth plus 0.5% dextrose and 3% glycerol, respectively. The organisms were grown at 37 C. for 4 days and then centrifuged in a Sharpless centrifuge at 40,000 revolutions per minute. The organisms so obtained were washed several times in distilled water to remove the phosphates, and were then transferred to a beaker. Because of the high speed of the centrifuge the mass of organisms so obtained was wet but not watery, and the water present was held in the pores of the caseous mass. Redistilled acetone, boiling point at 57 to 60 C., was added to the cheeselike mass of bacteria as they were obtained from the centrifuge, and the mixture was stirred briskly and allowed to stand for from 3 to 5 days. The mixture was then transferred to large centrifuge tubes and centrifuged at high

¹³ Jour. Infect. Dis., 1919, 25, p. 41.

¹⁴ Proc. Soc. Exper. Biol. and Med., 1921, 19, p. 62.

speed, the supernatant fluid decanted, more acetone added and the process of extraction by standing was continued until the organisms had been treated for from 10 to 14 days. Then the process was repeated with redistilled ethyl ether. At first the organisms and extracts were evaporated down slowly and then dried in an oven at 80 C. However, this process of heating was soon abandoned because the residue often underwent changes in color, and the extracts after such heating would go into their respective solvents only with difficulty. So the vacuum desiccator was resorted to and the residue and extracts were dried over P_2O_5 in a partial vacuum to constant weights. In all instances the procedures were strictly quantitative.

Table 1 gives the medium, residue, ether and acetone extracts and the percentage of total extractives as obtained by dividing the sum of

TABLE 1

PERCENTAGE OF EXTRACTS FOR VARIOUS ORGANISMS WHEN GROWN IN DIFFERENT MEDIUMS

Organism	Medium	Acetone Extract in Grams	Ether Extract in Grams	Residue in Grams	Percentage of Total Extracts
<i>Bacillus coli communis</i>	Plain broth.....	0.3523	0.0680	4.3791	9.59
	½% dextrose broth.....	0.6325	0.042	7.419	9.09
	3% glycerol broth.....	1.9642	0.050	10.1046	19.9
<i>Staphylococcus albus</i>	Plain broth.....	0.1185	0.1775	3.712	7.97
	½% dextrose broth.....	0.1074	0.0424	1.968	7.6
	3% glycerol broth.....	0.0961	0.1090	0.5136	39.9
<i>Bacillus megatherium</i>	Plain broth.....	0.0915	0.0483	1.527	9.15
	½% dextrose broth.....	0.0635	0.0372	0.559	18.1
	3% glycerol broth.....	0.3755	0.1820	1.649	33.8
<i>Bacillus mucosus</i>	Plain broth.....	0.4447	0.009	3.263	13.9
	½% dextrose broth.....	0.6735	0.0114	7.762	8.82
	3% glycerol broth.....	1.349	0.0140	8.839	15.42

the ether and acetone extracts by the residue. Acetone, because of its dehydrating properties, was used as the first solvent. It is true that acetone is a solvent for things other than fats and fatty acids, but we think it safe to assume that the amount of material other than fats weighed in each case will be in proportion to those weighed in any other case. None of the acetone or ether extractives from organisms grown on 0.5% dextrose broth gave any reduction on addition of Fehling's solution, so little if any sugar was extracted in any case. The ether was used mainly to preclude any possibility of having left any of the fat in the organism by acetone extraction.

From table 1 it appears that the percentage of extracts varies considerably for each organism when grown in different mediums. However, if we consider the biologic action of each organism on the

different mediums we will find a definite relationship. The colon bacillus has approximately the same percentage of extracts when grown on plain broth as on 0.5% dextrose broth, but has 100% more extracts when grown on 3% glycerol broth. The staphylococcus albus extracts approximate one another on plain broth and 0.5% dextrose broth but increase 500% on 3% glycerol. It will be recalled that both of these organisms ferment dextrose readily but do not ferment glycerol. The *B. megatherium*, which does not ferment either dextrose or glycerol, increases twofold in percentage of extracts when grown on 0.5% dextrose and fourfold when grown on 3% glycerol broth, as compared with plain broth. *B. mucosus*, which ferments both dextrose and glycerol readily, gives less extracts when grown on dextrose, and approximately the same amount on glycerol as on plain broth. It would seem, therefore, that the percentage of acetone and ether extracts will depend on whether or not the sugar or glycerol in the medium is fermented.

TABLE 2
RESULTS OF GROWTH OF *B. COLI-COMMUNIS* AND *B. COLI-COMMUNIOR* ON 1%
SACCHAROSE BROTH

Organism	Acetone Extract in Grams	Ether Extract in Grams	Residue in Grams	Percentage Extrac- tive
<i>Bacillus coli-communior</i>	0.5033	0.0030	5.885	8.60
<i>Bacillus coli-communis</i>	0.7813	0.0125	6.148	12.91

In order to study this point further pure strains of *B. coli-communis* and *B. coli-communior* were grown on 1% saccharose broth. The former does not ferment saccharose while the latter does. Table 2 gives the results obtained.

B. coli-communis, which gives 12.66% extractives, does not ferment the sugar but apparently metabolizes it into fat, while *B. coli-communior* does ferment the sugar, and the total extract is about 33% less.

In order to rule out the possibility of the acetone taking out material other than fats which would account for the results obtained, the acetone extracts from *Staphylococcus albus*, *B. mucosus* grown on plain broth, dextrose broth and glycerol broth, respectively, and *B. coli-communis* and *B. coli-communior* grown on 1% saccharose broth were reextracted with redistilled petroleum ether. The extracts of the other organisms were not so treated as they were not kept quantitatively and therefore would not give quantitative results. Petroleum ether will dissolve out the neutral fats and fatty acids. Table 3 gives the results.

A comparison of the total petroleum ether and ether extracts with the total acetone and ordinary ether extracts shows a proportionate reduction in each case. The petrol ether extracts represent more nearly the true fats or fatlike content of the organisms; the results are changed only in amount and not in proportion. Even though the other extracts were omitted in each case, the petrol ether extracts would give percentages in the same proportions as the figures given in table 3. Neither glycerol nor dextrose is soluble in petroleum ether so the results obtained are not due to the dissolving out of glycerol or dextrose from the medium.

Cramer's¹ work gives a 100% increase in ether and alcohol extractions for both *B. mucosus* and bacillus of rhinoscleroma when grown on

TABLE 3

RESULTS OF REEXTRACTING AND REDISTILLING ACETONE EXTRACTS FROM STAPHYLOCOCCUS ALBUS, *B. MUCOCOCCUS* GROWN ON PLAIN BROTH, DEXTROSE BROTH AND GLYCEROL BROTH

Organism	Medium	Petroleum Ether Extract in Grams	Ether Extract in Grams	Residue in Grams	Percentage of Total Ether Extracts	Percentage of Total Acetone and Ether Extracts (From Table 1)
Staphylococcus albus	Plain broth.....	0.0331	0.1775	3.712	5.68	7.97
	1/2% dextrose broth	0.0177	0.0424	1.968	3.06	7.6
	3% glycerol broth.	0.0358	0.1090	0.5136	28.19	39.9
Bacillus mucosus	Plain broth.....	0.1875	0.009	3.263	6.02	13.9
	1/2% dextrose broth	0.2386	0.0114	7.762	3.22	8.82
	3% glycerol broth..	0.2956	0.0140	8.839	3.52	15.42
B. coli-communis	1% saccharose broth	0.406	0.0125	6.148	6.80	12.91 Table 2
B. coli-communior	1% saccharose broth	0.2378	0.003	5.885	4.09	8.60 Table 2

5% dextrose as compared with 1% peptone. This is in contrast with the results herein recorded in the case of *B. mucosus*, in which it was found that the fat content did not increase on addition of carbohydrate to the medium. His results with the bacillus of rhinoscleroma, however, agree with our work since this organism does not ferment dextrose, and it would be expected that the fat content would increase when the organism is grown on this particular carbohydrate.

The work of Frouin may be cited here in support of our theory. The human strain of *B. tuberculosis* ferments glycerol, as shown by Theobald Smith,¹⁵ while the bovine and avian strains do not. Frouin's

¹⁵ Jour. Med. Research, 1904, 8, p. 253.

figures show from 42 to 45% fats for the bovine and 40% for the avian strains, but only 19.59% for the human strain. It would be interesting to know whether or not the one particular bovine strain in which he obtained approximately the same amount of fat (20%) as with the human strain, was a glycerol fermenter. Apparently the fat content of the tubercle bacillus grown on glycerol mediums does not depend on its virulence but rather on whether or not the glycerol is fermented.

Table 1 shows that *B. coli*, *B. megatherium* and *Staph. albus*, when grown on 3% glycerol, have a fat content approximating that of the tubercle bacillus. This naturally raises the question whether those organisms high in fat content are necessarily acid-fast. Investigation of this point revealed that their staining properties were not changed by the increased fat content. Therefore, the tubercle bacillus probably

TABLE 4
RESULTS OF GROWTH OF ORGANISMS ON PLAIN BEEF BROTH, DEXTROSE BROTH AND 3% GLYCEROL BROTH

	Plain Broth	½% Dextrose	3% Glycerol
<i>B. dysenteriae</i> (Flexner).....	—	— (f)	P
<i>B. dysenteriae</i> (Shiga).....	—	— (f)	?
<i>B. paratyphosus</i> B.....	—	— (f)	P
<i>B. paratyphosus</i> A.....	—	— (f)	P
<i>B. coli-communis</i>	—	— (f)	P
<i>B. coli-communior</i>	—	—	P
<i>B. mucosus-capsulatus</i>	P	P (f)	P (f)
<i>B. typhosus</i>	—	— (f)	P
<i>B. megatherium</i>	—	? (f?)	P
<i>Staphylococcus albus</i>	?	? (f)	P

P = Pellicle formation; ? = ring formation; — = no pellicle; (f) = ferments medium; (f?) = questionable fermentation.

does not owe its acid-fastness to its high fat content but rather to the character of the fats or other substances present.

In order to investigate further the theory on which this work was begun, namely, that the lack of wetting of organisms was due, in part, to an increase in their fat content, a series of organisms was grown on plain beef broth, 0.5% dextrose broth and 3% glycerol broth, respectively. Pure strains of *B. coli*, *B. typhosus*, *B. paratyphosus* A and B, *B. megatherium*, *B. dysenteriae*, *B. mucosus* and the *Staph. albus* were used (table 4).

B. mucosus forms a pellicle on all the mediums while none of the others form pellicles on plain broth or on 0.5% dextrose broth. However, on 3% glycerol broth they all form pellicles. From our studies on fat content we should predict that all the organisms of this series should have a high fat content when grown on 3% glycerol, with the

exception of the *B. mucosus*, since they do not ferment glycerol. If pellicle formation is a phenomenon of wetting, the lack of wetting, due to increased fat content of the organism when grown on certain mediums, might explain this phenomenon. That other factors such as the surface tension of the medium and inherent qualities of the bacteria themselves, undoubtedly enter into the causes of pellicle formation is conceded, because organisms such as *B. mucosus* form pellicles in spite of relatively low fat content, but the results we obtained would indicate that fat content of the organism is an important factor. The character of the fat present or the disposition of the fat in the organism may be the determining factor.

SUMMARY

Earlier workers have confined their investigations on the fat content of bacteria largely to the tubercle bacillus.

The results herein recorded seem to establish the general law that carbohydrates and glycerol are converted into fats or fatlike substances only when they are not fermented by the organisms.

The fat content of the tubercle bacillus bears no relation to the virulence of the organism but is determined by its biologic action on the glycerol in the medium.

The acid-fast staining properties of the tubercle bacillus are not due to its high fat content but probably to the character of the fats or other substances present.

Pellicle formation by bacteria is probably determined by the surface tension of the medium on the one hand and the fat content of the organisms on the other.

The methods of fat extraction used may have taken out substances other than fats or may not have extracted all the fats, but the results are comparable. In this paper we have called the extracts fats. Since there is no accepted criterion as to what a fat is, the term is used in its broadest sense.

THE VELOCITY OF FIXATION OF COMPLEMENT WITH BACTERIAL ANTIGENS

STUDIES ON COMPLEMENT FIXATION. VI

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In a recent paper from this laboratory,¹ it was shown that the velocity of fixation of complement with protein antigens and specific serums was not markedly affected by temperatures ranging from about 8 to 37 C. In these experiments, 2 vegetable proteins, edestin and phaseolin, of high purity were employed with immune rabbit serums. The temperatures of fixation were icebox (8-12 C.), room (18-22 C.) and water bath (37.5 C.) and the degree of fixation was measured at intervals extending from 5 minutes to 6 hours. These studies further indicated that the velocity of fixation of complement was directly proportional to the number of complement-fixing substances in the immune serum; that the greater part of complement was "fixed" during the first hour and that fixation was practically completed at the end of 4 hours.

More recently the velocity of fixation of complement in the Wassermann test was studied.² Six different extract antigens were employed. It was found that water bath, room, and icebox temperatures gave practically the same degree of fixation with all antigens studied except with one prepared according to Noguchi. The last antigen gave a somewhat higher degree of fixation at water bath than at icebox temperature. A fixation period of 4 hours at icebox temperature, however, was found to approach complete fixation of complement with all antigens, including the Noguchi.

The interest which has developed in recent years in complement fixation with bacterial antigens suggested the studies presented in this paper. It was desired to find to what extent different temperatures would affect the velocity of fixation of complement with bacterial antigens, and also the time and temperature of optimum fixation with different antigen-antibody complexes.

Received for publication July 15, 1922.

¹ Kahn, R. L.: Jour. Exper. Med., 1921, 34, p. 217.

² Kahn, R. L., and Olin, R. M., Jr.: Jour. Infect. Dis., 1921, 29, p. 630.

EXPERIMENTS

Antigens.—Six different antigens were employed. These were prepared from *B. typhosus*, *B. paratyphosus* A, *B. paratyphosus* B, *B. abortus*, *B. pullorum* and *B. mallei*, respectively. The organisms were obtained from stock cultures of this laboratory. These were first grown in broth for about 18 hours and then transferred on meat infusion agar of suitable P_H . The agar flasks were incubated from 24 to 48 hours, depending on the amount of growth. After incubation, the flasks were placed in the icebox over night. This, we believe, rendered it easier to wash off the organisms without breaking off particles of agar. To each flask were added 5 to 7 cc of normal salt solution and the growth washed off by means of a sterile cotton swab. The bacterial suspension was then placed in a graduated test tube and centrifuged at high speed. The

TABLE 1

VELOCITY OF FIXATION OF COMPLEMENT AT ICEBOX, ROOM, AND WATER-BATH TEMPERATURES

Fixation		Antityphoid Serum (C c)									No. of Positive Signs Denoting Degree of Fixation
Period, Min.	Temperature	0.01	0.007	0.004	0.003	0.002	0.001	0.0005	0.0003	0.0001	
0	1*	1	1	1	—	—	—	—	—	4
5	Water bath.	3	3	3	1	1	1	—	—	—	12
	Room.....	3	3	3	1	1	1	—	—	—	12
	Icebox.....	4	4	1	1	1	1	—	—	—	12
15	Water bath.	4	4	4	4	2	1	—	—	—	19
	Room.....	4	4	4	4	2	2	—	—	—	20
	Icebox.....	4	4	4	4	1	1	—	—	—	18
30	Water bath.	4	4	4	4	4	4	1	—	—	25
	Room.....	4	4	4	4	4	4	1	—	—	25
	Icebox.....	4	4	4	4	4	1	—	—	—	21
60	Water bath.	4	4	4	4	4	4	2	1	—	27
	Room.....	4	4	4	4	4	4	3	1	—	28
	Icebox.....	4	4	4	4	4	4	1	—	—	25
120	Water bath.	4	4	4	4	4	4	4	3	1	32
	Room.....	4	4	4	4	4	4	4	4	—	32
	Icebox.....	4	4	4	4	4	4	4	2	—	30
180	Icebox.....	4	4	4	4	4	4	4	4	—	32
240	Icebox.....	4	4	4	4	4	4	4	4	1	33
300	Icebox.....	4	4	4	4	4	4	4	4	1	33
300	Icebox.....	4	4	4	4	4	4	4	4	1	33

* 4 = + + + +, 3 = + + +, 2 = + +, 1 = +, and — = negative.

supernatant salt solution was pipetted off as completely as possible and 15 cc ether per 1 cc of packed organisms added. The tube was shaken vigorously and ether extraction permitted for 6 hours. At the end of this period, the ether was filtered off and the organisms were dried on the filter paper until no ether odor could be detected. The organisms were then resuspended in salt solution and titrated for their complement-binding, anticomplementary and hemolytic properties in the usual manner.

The employment of ether in connection with the preparation of bacterial antigens is highly desirable, according to one of us (Johnson). Ether extrac-

tion appears to reduce markedly the anticomplementary properties of these antigens. Bacterial antigens prepared by previous extraction with ether have been kept in the icebox in this laboratory for over a year, without acquiring anticomplementary properties.

Immune Serums.—With the exception of the case of *B. abortus* in which bovine serum was employed, the specific serums were obtained from rabbits. These animals were immunized with the various organisms in the usual manner and the serums employed after they were found to contain a sufficient number of antibodies.

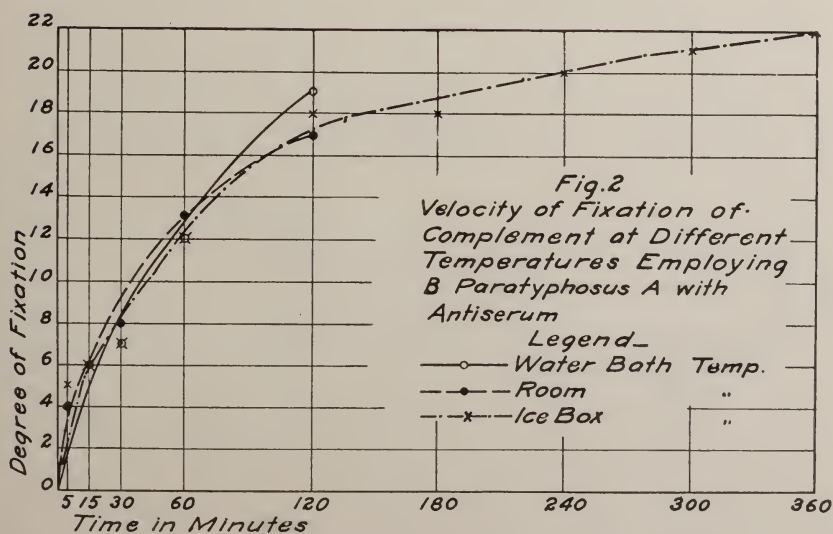
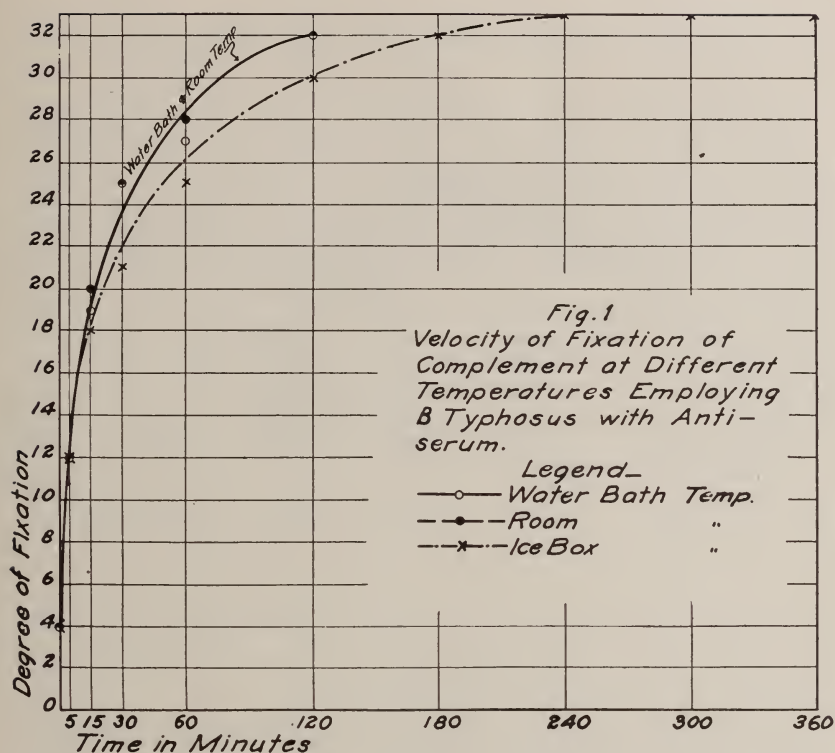
Complement-Fixation Tests.—These were carried out with a sheep cell system. All ingredients entering into the test were used in 0.1 cc quantities except the immune serums, which were graded from 0.01 to 0.0001 cc. Two units of amboceptor, 2 units of complement and from 2 to 3 units of the various antigens were employed. After a given fixation period, 0.1 cc of the standard 5% sheep cell suspension and 0.1 cc amboceptor (2 units) were added and incubated in the water bath (37.5 C.) for about 15 minutes, when the serum and antigen controls would be completely hemolyzed. All final readings were made after keeping the tubes in the icebox over night. The usual plus sign system was employed in recording the results.

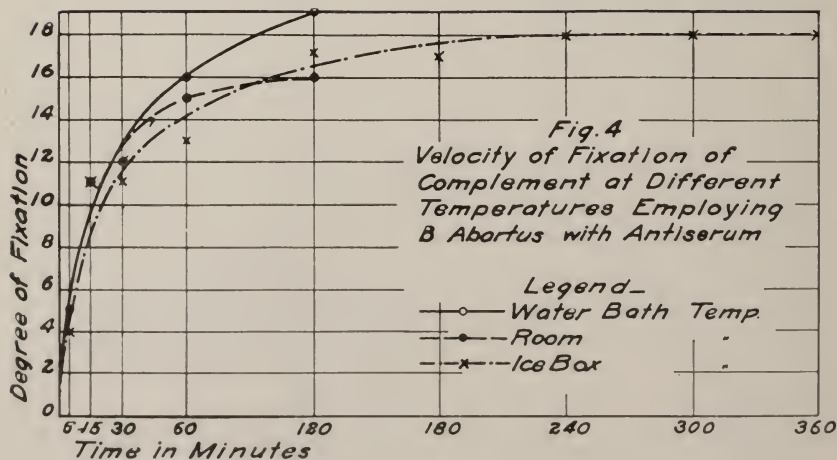
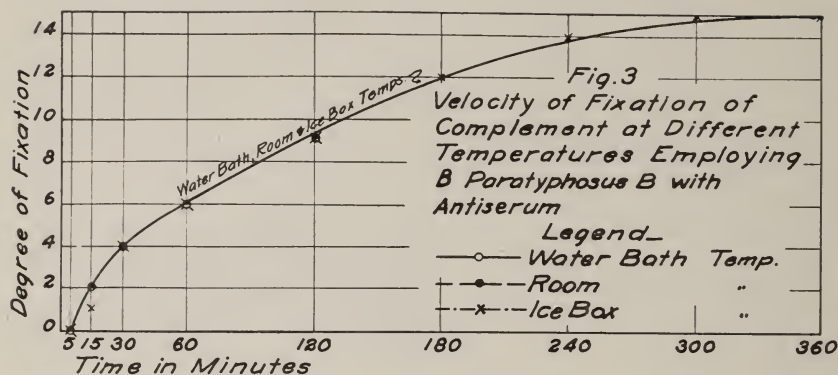
Table 1 gives an outline of the first experiment carried out with a typhoid antigen and antityphoid rabbit serum. The fixation periods were 0, 5, 15 and 30 minutes and 1, 2, 3, 4, 5 and 6 hours. It will be noted that the fixation periods at water-bath and room temperatures were not extended beyond 2 hours. This, because of marked deterioration of complement which takes place after prolonged exposure at these temperatures.

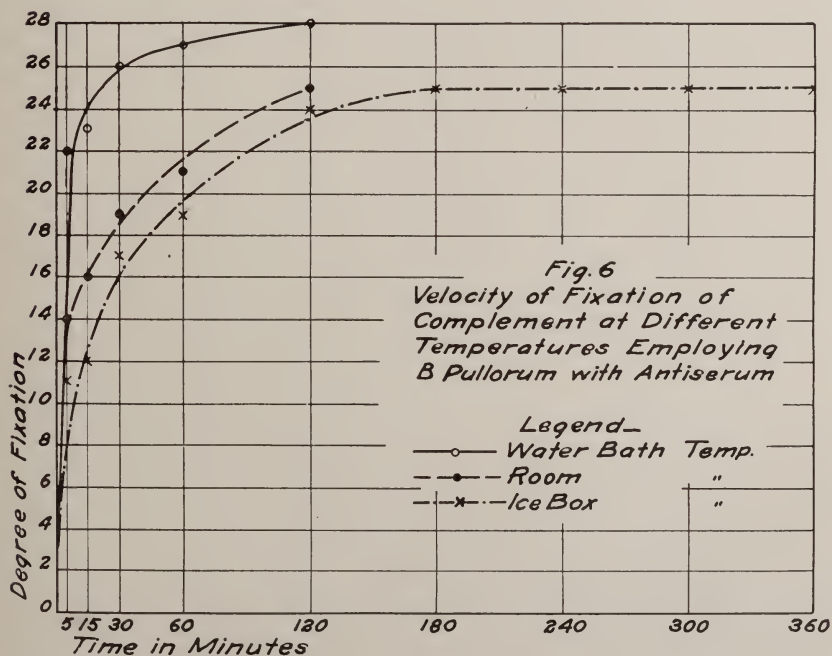
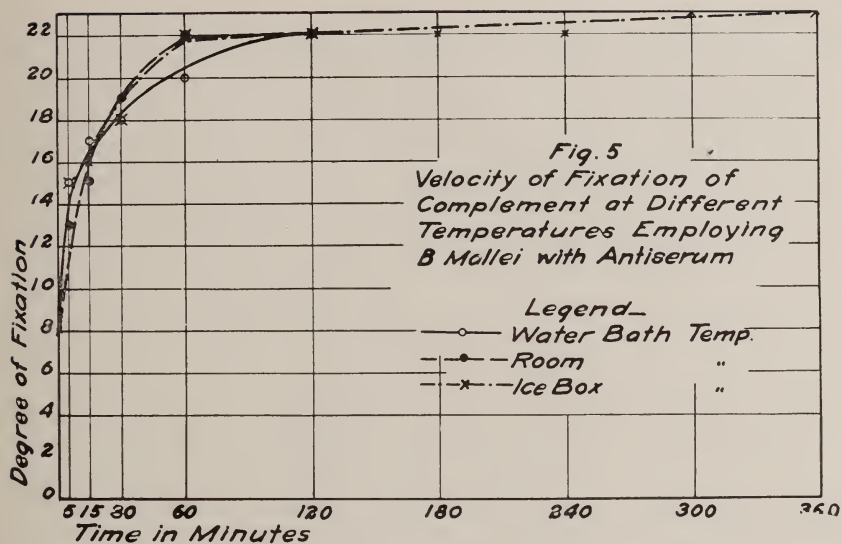
This table indicates that the velocity of fixation of complement with *B. typhosus* and antityphoid serum is somewhat greater at water-bath and room temperatures than icebox temperature. The difference, however, is comparatively small. Similar results have been obtained with *B. paratyphosus* A and B, *B. abortus* and *B. mallei* with their respective serums. *B. pullorum* gave considerably more fixation at water-bath than at icebox temperature. Figures 1 to 6 illustrate the findings with the various bacterial antigens. Although these represent single experiments, they may be considered as being representative of each antigen-antibody complex, the experiments in each case having been repeated no less than 3 times and in some cases as many as 10 times, with similar results.

DISCUSSION

A glance at the charts would indicate that a period of fixation of 1 hour in the water bath, generally employed in complement-fixation tests with bacterial antigens, does not represent a sufficient interval for maximum fixation in many cases. It is true that when employing







strongly positive serums a 1-hour period is quite ample; indeed, even less than 1 hour is sufficient in some instances. In the case of weak serums, however, one will frequently obtain no more than about 50% of fixation during this period. And the detection of weak complement-fixation reactions may, at times, prove to be of greater diagnostic value than the detection of strong reactions. The reason for this lies in the fact that strong complement-fixation reactions are usually accompanied by clinical manifestations of a disease, whereas in the case of weak reactions, the laboratory finding may serve as the only clue in establishing the presence of the disease. A fixation period limited to 1 hour, therefore, is likely to weaken just those reactions which ought to be rendered as sensitive as possible.

That a period limited to 1 hour is insufficient for complete fixation of complement in the Wassermann test, has been shown in a previous paper.² It was further shown that a period of 4 hours at ordinary icebox temperature appears to approach maximum fixation of complement in that test. Kolmer,³ in his recently standardized complement-fixation test for syphilis, requires a fixation period of 18 hours in the icebox. It is not unlikely that the difference between the complement-fixation procedure of Kolmer and that of this laboratory explains in part our different conclusions with regard to the period of fixation. Although we are inclined to think that 18 hours is more than is needed for complete fixation of complement in the Wassermann test, it would appear, both from the studies of Kolmer and our own, that a fixation period of 1 hour is quite insufficient for these tests. And there is no reason to believe that the inter-reaction of complement with syphilitic serum and extract antigen is essentially different from the inter-reaction of this ingredient with specific antigen-antibody complexes.

Furthermore, whenever it is experimentally established that the velocity of fixation of complement with a given antigen-antibody mixture goes on equally as well at water bath and icebox temperatures, the latter temperature is to be preferred. The reason for this is that complement deteriorates at water bath temperature, whereas it is practically preserved in the icebox. It may be of interest, in this connection, to record the following observation made during the course of these fixation experiments. On adding standard amounts of sheep cells and hemolysin to a series of tests after 1-hour fixation periods at different temperatures, it was observed again and again that the serum and antigen controls hemolyzed far more readily in the tests in which fix-

² *Jour. of Syphilis*, 1922, 6, p. 82.

tion was carried out in the icebox than in those in which fixation was carried out in the water bath. The explanation for this is clear. In the tests in which fixation was carried out in the icebox, the complement was practically as potent as in the beginning of the experiment and hemolysis of the serum and antigen controls took place readily. In the tests which were "fixed" in the water bath, however, the controls hemolyzed slowly because the complement had somewhat deteriorated during fixation. This explains also why slightly stronger reactions are frequently observed after 2 hours' fixation in the water bath compared with the same period in the icebox, as indicated in some of the charts. The stronger reactions are undoubtedly due in a large measure to complement deterioration rather than to specific fixation. It is evident, since the disappearance of complement forms the underlying basis of complement-fixation tests, that every factor which tends to destroy complement is to be eliminated from this test.

Assuming that a 1-hour period of fixation at water bath temperature is not conducive to dependable complement-fixation results with bacterial antigens, the question arises, What should be the underlying basis for correct fixation with these antigens? Judging from our results, it would appear that with 5 out of the 6 organisms studied a fixation period of 4 hours at ordinary icebox temperature approaches complete fixation of complement and may be taken as a reasonably safe procedure. Aside from the fact, however, that our studies are limited to this small number of organisms, it is conceivable that even with these organisms different complement fixation procedures might affect the phenomenon of fixation. We would say, therefore, when undertaking a series of complement-fixation studies with a given bacterial antigen, that the optimum time and temperature of fixation should be determined in that case in the same way as other optimum conditions of the test are determined. This can be accomplished readily by carrying out several experiments as outlined in table 1.

For correct complement-fixation tests, we insist on titrating a number of ingredients, and there is no reason why we should not also determine the proper mode of fixation by special titration. Our present criteria for fixation of complement with bacterial antigens are based largely on empirical data obtained with several strongly positive and negative serums. What is needed is some quantitative measurements of fixation at different temperatures carried out with different dilutions of positive serum and, in our opinion, the outline indicated in table 1 answers the purpose. One may leave out the 0, 5 and 15

minute fixation periods from this velocity experiment. But the other phases of this outline should be adhered to. Three different immune serums tested with a given bacterial antigen as outlined in the table should indicate the optimum mode of fixation with that antigen.

It should not be assumed that the attempt to find the optimum time and temperature of fixation with each bacterial antigen will lead to marked variations in the method of fixation on the part of different workers. On the contrary, judging from our complement-fixation studies with protein, Wassermann, as well as bacterial, antigens, we are inclined to believe that different workers will, in most cases, approach the same method of fixation. We believe also that icebox fixation, in many cases, will be found to be superior to water-bath fixation. The period of fixation, however, will have to be judged in each case by the nonspecific complement-binding properties of the bacterial antigen resulting from prolonged incubation. If, for example, after 4 hours of fixation in the icebox there is a tendency for a given bacterial antigen to be anticomplementary, the fixation period may have to be limited to 2 hours. This fixation period would be found to be more than ample with strong serums and would assure about 80% of fixation with weak serums.

SUMMARY AND CONCLUSIONS

The velocity of fixation of complement at icebox, room and water-bath temperatures was studied with the following 6 antigen-antibody complexes: *B. abortus* with specific bovine serum, *B. typhosus*, *B. paratyphosus* A and B, *B. mallei* and *B. pullorum* with specific rabbit serum. The fixation periods in each case were 0, 5, 15 and 30 minutes and 1, 2, 3, 4, 5 and 6 hours.

It was observed that the velocity of fixation of complement is practically the same at the different temperatures studied. This was true with all organisms except *B. pullorum*. With this organism, fixation at water-bath temperature was more marked than at icebox and room temperatures.

It was further observed that with the exception of *B. pullorum*, a fixation period of 4 hours at ordinary icebox temperature is superior to a 1-hour period in the water bath. This period of fixation in the water bath was found to be of insufficient length to bring about complete fixation of complement with 5 of the organisms studied. Furthermore, the employment of water bath temperature for fixation of complement should be avoided as far as possible because this temperature hastens the deterioration of complement.

Finally, it was pointed out that the mode of fixation of complement with a given bacterial antigen should be determined by special "titration" in the same way that various other factors of the complement-fixation test are determined by titration. The numerous ways of preparing bacterial antigens combined with the varying complement-fixation procedures employed by different workers, in our opinion, necessitates this step until we learn more of the laws governing the phenomenon of complement fixation. A simple outline for determining the optimum temperature and time of fixation of complement with different bacterial antigen-antibody complexes is indicated.

THE DETERMINATION OF THE OPTIMUM AMOUNT OF ANTIGEN IN COMPLEMENT-FIXATION TESTS *

STUDIES ON COMPLEMENT FIXATION. VII

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While studying the quantitative relation between serum and antigen in Wassermann as well as specific complement-fixation tests, data was obtained which suggested a simple procedure for determining the optimum amount of antigen for these tests. In this paper we shall briefly discuss the present method of titrating antigens for complement-fixation tests, and we shall outline the proposed method based on the newer observations.

INTRODUCTION

In the report of the Medical Research Committee (Great Britain) on the Wassermann test,¹ the following three procedures were suggested for the purpose of determining more closely the strength of the Wassermann reaction of a given serum:

"(1) To test the serum against diminishing quantities of extract (antigen), keeping the amounts of the other ingredients the same.

"(2) To use diminishing amounts of serum, keeping the amounts of the other ingredients the same.

"(3) To test the serum against increasing quantities of complement, such as 3, 5, 10 and 15 doses."

Judging from procedures (1) and (2), it would appear that there exists a direct proportional relation between serum and antigen in the Wassermann test; that decreasing serum, keeping the antigen constant or decreasing antigen, keeping the serum constant, gives similar results. The wide acceptance of this view is indicated by the fact that most serologists recommend the employment of proportionally large amounts of antigen in complement-fixation tests, the assumption being that when the amounts of the various ingredients are kept the same, the larger the amount of antigen employed the stronger the reaction.

Three "titrations" form, at the present time, the basis for standardizing a given antigen for complement-fixation tests. The first titration

Received for publication July 15, 1922.

*For preliminary report see *Proceed. Soc. for Exper. Biol. and Med.*, 1921, 19, p. 128.

¹ The Wassermann Test: Special Report, Series No. 14 of the Medical Research Committee, H. M. Stationery Office, London, 1918, p. 41.

has for its aim the determination of the antigenic unit or the smallest quantity of antigen which will produce complete fixation of complement in the presence of a given amount of positive serum in some standardized complement-fixation technic. The second titration determines the smallest quantity of antigen which is capable of binding complement in the absence of positive serum (anticomplementary unit), and the third titration, the smallest quantity which is capable of exerting any lytic properties on red cells (hemolytic unit). Based on the results of these titrations, one aims to employ in the tests a quantity of antigen which should contain as many antigen units as possible and at the same time be neither anticomplementary nor hemolytic in at least twice this quantity.

It is obvious that an antigen employed in complement-fixation tests should be as free as possible from anticomplementary and hemolytic properties. Whether or not, however, it can be accepted as a general rule that a larger number of antigen units represents a higher degree of sensitiveness is, in our opinion, questionable. Coca and L'Esperance² have indeed shown in the case of the Wassermann test that a smaller amount of lipoid antigen may, in some cases, give a stronger Wassermann reaction than a larger amount of the same antigen. And yet it is widely accepted that a high degree of concentration of antigen is essential for a high degree of sensitiveness in this reaction. Thus, Ottenberg,³ who has recently studied various methods for determining the optimum amount of antigen for the Wassermann reaction, sums up his paper by saying: "In the Wassermann systems in which the dose of complement is either fixed (0.1 c.c.) or is two hemolytic units, . . . the more one can increase the antigen dose (and still remain below the point where nonspecific fixations are obtained) the better the results." It should be stated that in the complement-fixation system referred to in this paper, 2 hemolytic units of complement are employed.

In complement fixation with bacterial antigen the same general view prevails, namely, that the more antigen one employs in a given test the more sensitive the reaction, providing the quantity of antigen used is free from anticomplementary and hemolytic properties.

Considered from a purely theoretical point of view, the method of determining antigen units by titration with strongly positive serums is open to question. Since large variations exist in the complement-binding power of different positive serums, an antigen unit obtained

² Jour. Immunol., 1916, 1, p. 129.

³ Ibid., 1917, 2, p. 47.

with one positive serum may not be the same unit that would be obtained with a serum of different potency. Thus, it is well known that an antigen unit obtained with a serum which approaches 400 plus will contain considerably less antigenic substance than the unit obtained with a serum which just approaches 4 plus. In other words, although the implication when speaking of an antigen unit is that we are dealing with a relatively constant quantity, it may show wide variations depending on the potency of the serum employed in the determination of the unit. Furthermore, in employing a relatively large number of units, we are employing an empirical quantity rather than one which conforms with the needs of the serums tested.

Our aim should be in a given antigen titration, not to find the smallest amount which will produce fixation with a strongly positive serum and then arbitrarily use many times this amount, but rather to find, if possible, the amount of antigen which will be capable of rendering the complement-fixation reactions as sensitive as possible. Fortunately, strongly positive serums are not markedly affected by the amount of antigen employed. These serums will give strong reactions with quantities of antigen which are considerably removed from the optimum range of fixation. The problem thus narrows itself down to the determination of the amount of antigen which will be capable of enhancing the reactions with serums of moderate or weak potency. This problem forms the underlying basis of the studies reported in this paper.

EXPERIMENTS

An attempt was first made to study the quantitative relation between serum and antigen in complement-fixation tests. It was desired to find to what extent serum and antigen might be replaced by one another and still obtain the same results. Three Wassermann and three bacterial antigens were employed in these experiments. The former antigens consisted of an alcoholic extract of dried beef heart muscle previously freed from ether soluble lipoids; the same antigen, fortified with 0.4% cholesterol and a Noguchi antigen prepared from dried beef heart. The bacterial antigens consisted of *B. abortus*, *B. mallei* and *B. typhosus*. The complement-fixation tests were carried out as outlined in the earlier papers of this series.

The following is an outline of the first experiment, as recorded in table 1 (serum 1). A serum giving a + + + + reaction with the Wassermann test was inactivated for one-half hour at 56 C. and diluted with salt solution in the proportion of 1:10. The diluted serum was pipetted into 6 rows of 10 Wassermann tubes as follows: The first row received 0.01 cc of diluted serum, the second row, 0.02 cc, the third 0.03 cc, the fourth 0.05 cc, the fifth, 0.07 cc and the sixth, 0.1 cc of diluted serum. Alcoholic extract antigen was diluted with salt solution in the proportion of 1:50 and the mixture pipetted into each of the 6 rows of tubes. The amounts ranged from 0.01 to 0.3 cc. One-tenth cc of complement, equivalent to 2 units, was added to each

tube and sufficient amount of salt solution to make a total quantity of 0.3 c c in each case. Fixation was carried out in the icebox for 4 hours. After this period 0.1 c c sheep cell suspension (5%) and 0.1 c c of amboceptor solution (2 units) were added and incubated for about 15 minutes in the water bath. At the end of this period the rack of tubes was placed in the icebox over night. The readings were recorded the following morning.

An examination of table 1 reveals several interesting phases regarding the relation of serum and antigen in complement-fixation. One may consider the 0.01 and 0.02 c c quantities of serum (serum I) as having insufficient reagin for complement binding and therefore as corresponding to negative serums. As the serum approaches the 0.1 c c quantity,

TABLE 1
AMOUNT OF ANTIGEN GIVING STRONGEST COMPLEMENT FIXATION REACTIONS

Syphilitic Serum		Alcoholic Antigen Prepared from Beef Heart, C c									
No.	C c	0.01	0.02	0.04	0.06	0.08	0.1	0.15	0.2	0.25	0.3
I	0.01	—*	—	—	—	—	—	—	—	—	—
	0.02	—	—	—	—	—	—	—	—	—	—
	0.03	—	—	±	±	2	2	1	1	—	—
	0.05	—	—	1	1	3	3	3	2	2	±
	0.07	—	—	1	4	4	4	4	4	4	4
	0.1	—	—	2	4	4	4	4	4	4	4
II	0.01	—	—	—	—	—	—	—	—	—	—
	0.02	—	—	±	1	3	3	1	1	1	±
	0.03	—	—	1	2	3	3	3	3	3	2
	0.05	—	±	1	3	4	4	4	4	4	4
	0.07	—	±	1	3	4	4	4	4	4	4
	0.1	—	±	1	3	4	4	4	4	4	4
III	0.01	—	—	—	—	—	—	—	—	—	—
	0.02	—	—	—	—	—	—	—	—	—	—
	0.03	—	—	—	—	—	—	—	—	—	—
	0.05	—	—	1	1	1	1	—	—	—	—
	0.07	—	1	1	2	3	3	3	2	2	2
	0.1	±	3	3	4	4	4	4	4	3	3

* In this and subsequent tables, 4 = + + + +, 3 = + + +, 2 = + +, 1 = +, and — = negative.

it gradually gains in complement-binding power. It thus appears that the higher the concentration of complement-fixing substances in the serum the stronger the reaction. In other words, there is a direct relation between the quantity of serum employed and the strength of the reaction, which, indeed, is well established. When turning to the antigen, we find that antigenic potency and strength of the final reaction are not directly proportional to one another, except in a limited degree. This reagent appears to have a narrow range of optimum fixation for serums of weak or moderate potency. Strongly positive serums do not seem to be markedly affected by the amounts of antigen employed; quantities which are considerably removed from the optimum binding range will

nevertheless give 4 + reactions. In the case of weak serums, however, the amount of antigen employed appears to play an important part in the final outcome of the reaction. If a quantity of antigen outside of the optimum range is employed, such serums may show little or no complement-binding powers. According to the findings recorded in table 1, the optimum antigen range with each of the 3 serums tested is from 0.08 to 0.1 c c. It is apparent that only within this range will one obtain most sensitive reactions with this antigen.

It is of interest to note that the results obtained with each of the 3 serums in table 1 indicate also the so-called antigen unit, or smallest amount which gives complete fixation of complement with serums of

TABLE 2
AMOUNT OF ANTIGEN GIVING STRONGEST COMPLEMENT-FIXATION REACTIONS

Syphilitic Serum		Cholesterinized Antigen Prepared from Beef Heart, C c									
No.	C c	0.01	0.02	0.04	0.06	0.08	0.1	0.15	0.2	0.25	0.3
I	0.01	—	—	—	—	—	—	—	—	—	—
	0.02	—	—	—	—	—	—	—	—	—	—
	0.03	—	—	—	1	2	1	±	±	—	—
	0.05	—	—	1	4	4	4	3	3	1	±
	0.07	—	1	3	4	4	4	4	4	4	4
	0.1	—	1	3	4	4	4	4	4	4	4
II	0.01	—	—	—	—	—	—	—	—	—	—
	0.02	—	—	—	—	—	—	—	—	—	—
	0.03	—	—	—	—	—	—	—	—	—	—
	0.05	—	—	—	1	1	1	—	—	—	—
	0.07	—	—	1	3	3	2	2	2	2	1
	0.1	—	—	2	4	4	4	4	4	3	2
III	0.01	—	—	1	1	2	2	1	1	1	—
	0.02	—	—	2	2	4	3	3	2	1	—
	0.03	—	±	2	3	4	4	4	3	3	3
	0.05	—	±	3	4	4	4	4	4	4	4
	0.07	—	±	3	4	4	4	4	4	4	4
	0.1	—	1	3	4	4	4	4	4	4	4

high potency. Thus, in the case of serums I and III, the unit of antigen is 0.06 c c; in serum II, 0.08 c c. The fact that the optimum amount of antigen approaches the antigen unit, would indicate that the amount of antigen which is highly sensitive with weak serums does not necessarily consist of a large number of units. Two units, or better still 1½ units, would seem to be the amount of antigen for sensitive reactions. It is not unlikely that many of the difficulties which occur from time to time in connection with complement-fixation tests are due to the employment of relatively large amounts of antigen. For, aside from the inhibitory effect of such antigen quantities on some weakly positive serums, foreign elements are introduced which are capable of nonspecific fixation of complement.

Tables 2 and 3 give the results obtained with a cholesterinized and Noguchi antigen. Both of these antigens were diluted 1:75 with normal salt solution. These dilutions were based on previous experience gained with these antigens. The amount of salt solution to be employed for diluting a given extract antigen before titrating it depends largely on the way the antigen has been prepared. Thus, an extract prepared from wet heart muscle might be diluted 1:10 to 1:30 with saline, whereas if the extract is prepared from dried muscle, it might be diluted about 1:50 to 1:75. A glance at table 2 and 3 will indicate that the findings obtained with the cholesterinized and Noguchi antigens are practically the same as those obtained with the alcoholic antigen.

TABLE 3
AMOUNT OF ANTIGEN GIVING STRONGEST COMPLEMENT-FIXATION REACTION

Syphilitic Serum		Noguchi Antigen Prepared from Beef Heart, C c									
No.	C c	0.01	0.02	0.04	0.06	0.08	0.1	0.15	0.2	0.25	0.3
I	0.01	—	—	—	—	—	—	—	—	—	—
	0.02	—	—	±	1	1	1	±	±	—	—
	0.03	—	—	1	1	1	1	1	±	—	—
	0.05	—	1	1	1	2	2	1	1	1	1
	0.07	—	1	2	3	3	3	3	3	2	2
	0.1	—	2	4	4	4	4	4	4	4	4
	0.1	—	2	4	4	4	4	4	4	4	4
II	0.01	—	—	—	—	—	—	—	—	—	—
	0.02	—	—	1	1	1	1	1	±	—	—
	0.03	1	1	2	2	3	3	2	2	1	1
	0.05	—	—	2	2	3	3	3	2	2	1
	0.07	1	1	2	2	3	3	3	3	3	2
	0.1	1	2	3	4	4	4	4	4	4	4
	0.1	1	2	3	4	4	4	4	4	4	4
III	0.01	—	—	—	1	1	1	1	1	—	—
	0.02	—	—	2	2	4	4	4	4	4	1
	0.03	—	—	4	4	4	4	4	4	4	4
	0.05	2	3	4	4	4	4	4	4	4	4
	0.07	2	3	4	4	4	4	4	4	4	4
	0.1	2	3	4	4	4	4	4	4	4	4
	0.1	2	3	4	4	4	4	4	4	4	4

From 0.08 to 0.1 c c appears to be the optimum amount of antigen in both cases.

It should be stated that any procedure for finding the optimum amount of antigen will necessarily apply only to a given complement-fixation system. These tables, therefore, should be looked on as suggestive outlines only. Different workers will carry out different antigen titrations depending on their complement-fixation technic, just as different workers titrate complement differently although for the same end. There will be workers, for example, who will first determine the antigen unit in the usual manner and then by a simple titration find the number of units giving optimum results with weak serums. Then again

there may be workers who, although following the original outline, will not find it necessary to employ 6 rows of tubes in a given antigen titration, but instead, limit themselves to 3 rows. The important point to keep in mind is that it is desirable not to find the amount of antigen which will give fixation with a serum which is rich in complement fixing substances, but rather with a serum which contains as few of these substances as possible.

When turning to specific complement-fixation tests carried out with bacterial antigens, we find, as was true in the Wassermann test, that there is a direct relation between the quantity of positive serum employed and the strength of the reaction. In the case of the antigen,

TABLE 4
RELATION OF SERUM TO ANTIGEN IN SPECIFIC COMPLEMENT-FIXATION TESTS

Immune Bovine Serum		B. abortus Antigen, C c									
No.	C c	0.01	0.02	0.04	0.06	0.08	0.1	0.15	0.2	0.25	0.3
I	0.01	—	—	—	—	—	—	—	—	—	—
	0.02	—	—	—	—	—	—	—	—	—	—
	0.03	—	—	—	—	—	—	—	—	—	—
	0.05	—	—	+	2	3	4	4	4	4	4
	0.07	—	—	2	2	3	4	4	4	4	4
	0.1	—	—	2	2	4	4	4	4	4	4
II	0.01	—	—	+	2	3	4	4	4	4	4
	0.02	—	—	+	3	4	4	4	4	4	4
	0.03	—	—	+	3	3	4	4	4	4	4
	0.05	—	—	+	3	3	4	4	4	4	4
	0.07	—	—	2	2	4	4	4	4	4	4
	0.1	—	—	2	2	4	4	4	4	4	4
III	0.01	—	—	—	1	2	2	2	3	3	3
	0.02	—	—	1	2	2	3	4	4	4	4
	0.03	—	—	1	2	3	4	4	4	4	4
	0.05	—	—	1	2	3	4	4	4	4	4
	0.07	—	1	2	3	4	4	4	4	4	4
	0.1	—	1	2	3	4	4	4	4	4	4

however, we do not find an optimum range of fixation analogous to that shown by the Wassermann antigens. Furthermore, increasing the amount of bacterial antigen keeping the serum constant has only a limited effect on the final results. Thus, an amount of serum giving, let us say, a 2+ reaction with 0.05 c c of antigen, will be likely to give the same reaction with as much as 0.3 c c of antigen.

The first experiments were carried out with an abortion antigen and 3 immune bovine serums. The antigen was diluted 1:30 with salt solution and the titration carried out as with the extract antigens and syphilitic serums. A glance at table 4 will reveal that increasing the quantity of serum increases the strength of the reactions, whereas

increasing the quantity of antigen affects the reactions in only a limited degree. It will be noted also, as in the case of the Wassermann reaction, that weak serums are far more susceptible to the antigen quantity than strong serums, the latter showing marked fixation with a wide range of antigen quantities.

TABLE 5
RELATION OF SERUM TO ANTIGEN IN SPECIFIC COMPLEMENT-FIXATION TESTS

Immune Rabbit Serum		B. mallei Antigen, C c									
No.	C c	0.01	0.02	0.04	0.06	0.08	0.1	0.15	0.2	0.25	0.3
I	0.01	—	—	—	—	—	—	—	—	—	±
	0.02	—	—	—	—	—	—	—	—	—	±
	0.03	—	—	—	1	3	4	4	4	4	4
	0.05	—	—	1	2	3	4	4	4	4	4
	0.07	—	—	1	2	3	4	4	4	4	4
	0.1	—	±	1	3	3	4	4	4	4	4
II	0.01	—	—	—	1	1	1	1	1	1	1
	0.02	—	—	1	3	3	3	3	3	3	3
	0.03	—	—	1	4	4	4	4	4	4	4
	0.05	—	—	1	4	4	4	4	4	4	4
	0.07	—	1	3	4	4	4	4	4	4	4
	0.1	—	1	3	4	4	4	4	4	4	4
III	0.01	—	—	—	—	—	—	—	—	—	—
	0.02	—	—	—	—	—	—	—	—	—	—
	0.03	—	—	—	1	1	1	1	2	2	2
	0.05	—	—	1	2	4	4	4	4	4	4
	0.07	—	—	3	3	4	4	4	4	4	4
	0.1	—	—	3	4	4	4	4	4	4	4

TABLE 6
RELATION OF SERUM TO ANTIGEN IN SPECIFIC COMPLEMENT-FIXATION TESTS

Immune Rabbit Serum		B. typhosus Antigen, C c									
No.	C c	0.01	0.02	0.04	0.06	0.08	0.1	0.15	0.2	0.25	0.3
I	0.01	—	—	—	—	—	—	—	—	—	—
	0.02	—	—	—	—	—	—	—	—	—	—
	0.03	—	—	1	1	1	1	1	1	1	1
	0.05	—	±	2	2	2	2	2	2	2	2
	0.07	—	1	3	3	3	3	3	3	3	3
	0.1	—	2	4	4	4	4	4	4	4	4
II	0.01	—	—	1	1	1	1	1	1	1	1
	0.02	—	—	1	2	2	2	2	2	2	2
	0.03	—	—	1	2	2	2	2	2	2	2
	0.05	—	±	2	3	3	3	3	3	3	3
	0.07	—	1	4	4	4	4	4	4	4	4
	0.1	—	2	4	4	4	4	4	4	4	4

The same general findings were obtained with glanders and typhoid antigens and specific rabbit serums. These antigens were diluted 1:30 with salt solution and the titrations carried out as in the previous cases. Slightly stronger reactions due to increasing the antigen quantities are observed here and there, but, as a whole, the effect is comparatively small.

A glance at tables 4, 5 and 6 indicates that the amounts of bacterial antigen which are likely to give optimum results in complement-fixation tests are not as clearly defined as in the case of the alcoholic extract antigens. Indeed, there appears to exist no optimum range of fixation with these bacterial antigens. Yet, a titration of antigen such as outlined in these tables, seems to us capable of giving a better indication of the amount of antigen to employ in the tests than the old method of determining an antigen unit with some positive serum. The antigen quantity desired is the one giving most sensitive reactions with weakly positive serums and not an arbitrary number of antigen units. Assuming that 0.1 cc of abortion antigen approaches the most sensitive amount with serums of weak potency (table 4), we would say that the employment of 0.15 or 0.2 cc would be more than ample for complement-fixation tests. The same is true with the glanders and typhoid antigens. It would appear that increasing the amounts unduly would not necessarily render the reactions more sensitive. It might, on the other hand, increase nonspecific fixation of complement. It is not unlikely that the avoidance of excessive amounts of antigen in complement-fixation tests will tend to reduce the difficulties occasionally encountered due to anticomplementary and hemolytic properties of a given antigen, since these properties usually accompany high antigenic concentrations.

It should be stated also that the aim of the antigen titrations outlined in this paper is to find only the optimum quantities capable of producing the most sensitive complement-fixation reactions. These titrations refer therefore to the specific binding powers of antigen. The anticomplementary and hemolytic properties of a given antigen must be tested separately in each case.

DISCUSSION

The widely accepted view that serum and antigen are interchangeable in complement-fixation tests, is not entirely correct. Increasing serum, keeping the antigen constant, increases the strength of the reaction. But increasing antigen, keeping the serum constant, does not in the same degree increase the strength of the reaction. Indeed, the quantitative rôle of antigen in the Wassermann test appears to be different from its rôle in specific complement-fixation tests. In the former case we appear to have an optimum range of antigen capable of bringing forth most sensitive reactions. Increasing the amount of antigen beyond this range will result in extremely weak or negative

reactions. This particularly applies to serums of weak potency. In the case of specific complement-fixation tests, the antigens do not give any indication of possessing an optimum range of fixation. Increasing the amount of antigen will, up to a limited degree, increase the strength of the reaction; increasing the antigen beyond this degree does not seem to affect the reaction.

It is of interest that we have here another significant difference between complement fixation with bacterial antigens and specific serums and complement fixation with alcoholic extract antigens and syphilitic serums. The comparative thermostability of specific complement-fixing substances and thermolability of the similar substances present in the serum of syphilis, has been pointed out in an earlier paper of this series.⁴ It would appear that there exist inherent biologic differences between specific complement-fixation tests and the nonspecific test which is exemplified in the Wassermann reaction.

Coming back to the determination of the optimum amount of antigen in the Wassermann test, the following observation made by one of us in connection with a recently developed precipitation test for syphilis,⁵ appears to throw further light on this problem. This precipitation test essentially consists of mixing a given quantity of especially prepared and diluted extract antigen with 6 times the amount of syphilitic serum, and observing precipitates after incubation. While studying the quantitative relation between serum and antigen in this test, it was early observed that it is of the utmost importance to employ the proper amount of antigen and that an excess of this reagent inhibits the precipitation reaction, in many cases. Thus, a 6:1 proportion of serum to antigen gives good results. If the amount of antigen is gradually increased beyond this 6:1 proportion, weaker reactions are obtained. Equal parts of serum and antigen frequently inhibits the reaction. This is particularly true in the case of weak serums.

These observations are recorded because it appears to us not unlikely that the same phenomenon which tends to inhibit the precipitation reaction, when an excess of antigen is employed, inhibits also the complement-fixation reaction. The nature of this phenomenon, we are not, at the present time, prepared to explain. We feel, however, that the findings recorded in this paper with reference to the quantitative rôle of antigen in the complement-fixation reaction combined with the

⁴ Kahn, R. L.; Johnson, S. R., and Boyd, A. G.: *Jour. Infect. Dis.*, 1921, 29, p. 639.

⁵ Kahn, R. L.: *Arch. Dermat. & Syph.*, 1922, 5, pp. 570 and 734.

analogous findings in the precipitation reaction clearly indicate that an excessive amount of antigen should be especially avoided in complement fixation in syphilis.

With regard to complement fixation with bacterial antigens, large amounts of antigen, it appears, may also best be avoided, first, because such amounts are not necessary for sensitive reactions; second, because such amounts may lead to nonspecific fixation of complement.

SUMMARY

Studies on the quantitative relation between serum and antigen in the Wassermann as well as specific complement-fixation tests led to the involvement of a simple titration method for the determination of the optimum amount of antigen for these tests. The widely employed method of titrating antigen for complement-fixation tests consists of determining the so-called unit or smallest amount which will produce complete fixation of complement with a strongly positive serum and employing in the tests as many units as possible, providing the final amount is free from anticomplementary and hemolytic properties. This method is, in our opinion, based on the wrong assumption that the greater the concentration of antigen, keeping the other constituents the same, the stronger the reaction.

It was observed that with alcoholic extract antigens and syphilitic serums there exists an optimum range of antigen which will give complement-fixation reactions of high sensitiveness with comparatively weak serums. This optimum antigen range may be readily determined with each antigen by a simple titration indicated in the text. This titration also indicates that the employment of excessive amounts of antigen may render weak or moderate reactions, negative.

It was further observed that with bacterial antigens and specific immune serums similar antigen titrations did not disclose any optimum range of fixation for these tests. It could be readily discerned, however, that there is no advantage in employing excessive amounts of bacterial antigens. There appears, on the other hand, to be an important disadvantage since unnecessarily large amount of antigen may lead to nonspecific absorption of complement.

CONCLUSIONS

In titrating an antigen for complement-fixation tests, the aim should be to find that amount of antigen which will give reactions of high sensitiveness with weakly positive serums. Only these serums are

markedly affected by the amount of antigen; the strongly positive serums show complement-fixation powers with a comparatively wide range of antigen quantities.

The employment of excessive amounts of antigen in the Wassermann, as well as specific complement-fixation tests, should be avoided. Such amounts might lead to false negative reactions in the former test and nonspecific complement fixation in the latter tests.

THE EFFECT OF INACTIVATION ON COMPLEMENT-FIXING SUBSTANCES IN SYPHILITIC SERUM

STUDIES ON COMPLEMENT FIXATION. VIII

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It is generally accepted that when syphilitic serum is inactivated for one-half hour at 56 C., it loses some of its complement-binding substances. According to Noguchi,¹ a given serum may lose during the process of inactivation as much as 75% of its antibody content. It is not unlikely that many complement-fixation systems in syphilis which employ raw serum do so not to utilize the easily replaceable complement, but because of the general belief that a large percentage of syphilitic antibody is destroyed during inactivation. In order to overcome this antibody destruction, several workers have, in recent years, recommended a shortening of the inactivation period for the Wassermann test. Among these may be mentioned Simon² who employs a 10-minute period of inactivation and Kolmer³ who recommends in his newly standardized complement-fixation test, a 15-minute period.

Recent studies from this laboratory,⁴ however, indicate that the thermolability of syphilitic complement-fixing substances is apparent rather than real, and that in syphilis we are not dealing with true antibody destruction due to inactivation. It was observed that the mode of fixation employed in complement-fixation tests was frequently the determining factor as to whether or not complement-fixing substances disappeared as a result of heating. Thus, if the mode of fixation was 1 hour in the water bath, the effect of heating appeared to be, as a rule, a marked loss of complement-fixing substances. If, on the other hand, fixation was carried out for 4 hours at icebox temperature, the effect of heating resulted either in no loss or some gain in these substances, in most cases. Not being able to interpret this observation, it was felt that technical errors might have entered into it. It seemed worth while, therefore, to extend these studies to a comparatively large number of syphilitic serums in order to establish

Received for publication July 15, 1922.

¹ Serum Diagnosis of Syphilis, p. 97.

² Jour. Am. Med. Assn., 1919, 72, p. 1535.

³ Jour. of Syphilis, 1922, 6, p. 82.

⁴ Kahn, R. L.; Johnson, S. R., and Boyd, A. G.: Jour. Infect. Dis., 1921, 29, p. 639.

particularly whether the inactivation of serum for one-half hour at 56 C. is capable of bringing about an apparent increase in the number of complement-fixing substances over unheated serum.

It is frequently assumed that the purpose of inactivation is the destruction of native complement and the reduction of the anticomplementary properties of a given serum. Yet, in the precipitation reaction for syphilis recently proposed by one of us,⁵ which consists of mixing extract antigen with syphilitic serum and where complement apparently plays no rôle, inactivation nevertheless enhances the reaction. Furthermore, the reduction of anticomplementary properties of a given serum by heat does not apply to the precipitation reaction, as anticomplementary serums exert no special effect on this test. The question arises, therefore, if inactivation is capable of increasing the activity of the precipitation elements of the serum, might it not also be capable of increasing the activity of the complement-fixing substances of the same serum?

EXPERIMENTS

Altogether 100 serums were tested. Each serum was examined for its complement-fixing substances in a raw state and after inactivation for one-half hour at 56 C. The loss or gain in antibody content due to inactivation could thus be readily ascertained. The complement-fixation tests were carried out with a sheep cell system and guinea-pig complement, as in the preceding studies of this series. These tests were carried out in duplication, employing a 1-hour fixation period in the water bath at 37.5 C. in one case, and a 4-hour period in the icebox at 8-12 C. in the other. Two antigens were employed; an alcoholic extract of dried beef heart previously freed from ether soluble lipoids and a similar antigen fortified with 0.4% cholesterolin.

One of the first observations made in connection with these studies was that strongly positive serums do not seem to be influenced by inactivation. These serums show, in most cases, practically the same number of complement-fixing substances before and after inactivation. Neither are they markedly influenced by the mode of fixation. Whether the fixation period is 1 hour in the water bath or 4 hours in the icebox, the results are practically the same. This, it seems to us, is of some significance. It will be recalled that in the preceding paper⁶ it was shown that strongly positive serums are not markedly affected by the

⁵ Kahn, R. L.: *Arch. Dermat. & Syph.*, 1922, 5, pp. 570 and 734.

⁶ Kahn, R. L., and Johnson, S. R.: *Jour. Infect. Dis.*, 1922.

TABLE 1
DIFFERENCE IN COMPLEMENT-FIXATION POWER OF RAW AND INACTIVATED SYPHILITIC
SERUM. TESTS WITH ALCOHOLIC ANTIGEN OF BEEF HEART

Serum		Mode of Fixa- tion	Serum, C c								Total Plus Signs	Effect of Inactivation	
No.	Kind		0.01	0.007	0.004	0.003	0.002	0.001	0.0005	0.0001		Antibody	
												Loss %	Gain %
1	Raw	Water bath	4	4	1	1	—	—	—	—	10	60	
	Heated	Water bath	3	1	—	—	—	—	—	—	4		
	Raw	Ice-box	4	4	2	1	—	—	—	—	11	9	
	Heated	Ice-box	4	4	1	1	—	—	—	—	10		
2	Raw	Water bath	4	4	4	—	—	—	—	—	12	59	
	Heated	Water bath	4	1	—	—	—	—	—	—	5		
	Raw	Ice-box	4	4	4	1	1	—	—	—	14	7	
	Heated	Ice-box	4	4	4	3	2	—	—	—	15		
3	Raw	Water bath	4	4	4	1	1	—	—	—	14	35	
	Heated	Water bath	4	3	1	1	—	—	—	—	9		
	Raw	Ice-box	4	4	4	1	—	—	—	—	13	8	
	Heated	Ice-box	4	4	3	2	1	—	—	—	14		
4	Raw	Water bath	3	3	2	1	1	1	—	—	11	46	
	Heated	Water bath	2	2	1	1	—	—	—	—	6		
	Raw	Ice-box	4	4	2	2	1	—	—	—	13	28	
	Heated	Ice-box	4	4	3	3	2	2	..	—	18		
5	Raw	Water bath	4	4	3	2	1	1	—	—	15	100	
	Heated	Water bath	—	—	—	—	—	—	—	—	0		
	Raw	Ice-box	3	2	1	1	1	—	—	—	8	44	
	Heated	Ice-box	4	4	4	3	2	1	—	—	18		
6	Raw	Water bath	4	4	4	4	3	1	—	—	20	44	
	Heated	Water bath	4	4	2	1	—	—	—	—	11		
	Raw	Ice-box	4	4	4	4	2	1	—	—	19	21	
	Heated	Ice-box	4	4	4	2	1	—	—	—	15		
7	Raw	Water bath	—	—	—	—	—	—	—	—	0		
	Heated	Water bath	1	—	—	—	—	—	—	—	1		
	Raw	Ice-box	—	—	—	—	—	—	—	—	0		
	Heated	Ice-box	4	4	1	1	—	—	—	—	10		
8	Raw	Water bath	4	4	4	4	2	—	—	—	13	22	
	Heated	Water bath	4	4	4	1	1	—	—	—	14		
	Raw	Ice-box	4	4	4	4	1	—	—	—	16	6	
	Heated	Ice-box	4	4	4	4	1	—	—	—	17		
9	Raw	Water bath	4	3	1	—	—	—	—	—	8	57	
	Heated	Water bath	1	—	—	—	—	—	—	—	1		
	Raw	Ice-box	4	4	1	—	—	—	—	—	9	0	0
	Heated	Ice-box	4	4	1	—	—	—	—	—	9		
10	Raw	Water bath	4	4	2	—	—	—	—	—	10	10	
	Heated	Water bath	4	4	1	—	—	—	—	—	9		
	Raw	Ice-box	4	4	4	2	—	—	—	—	12	25	
	Heated	Ice-box	4	4	4	3	1	—	—	—	16		

The period of water bath fixation was 1 hour; of icebox fixation, 4 hours.
4 = +++++, 3 = +++, 2 = ++, 1 = +, and — = negative.

TABLE 2

DIFFERENCE IN COMPLEMENT-FIXATION POWER OF RAW AND INACTIVATED SYPHILITIC SERUM. TESTS WITH CHOLESTERINIZED ANTIGEN OF BEEF HEART

Serum		Mode of Fixa- tion	Serum, C c								Total Plus Signs	Effect of Inactivation	
No.	Kind		0.01	0.007	0.004	0.003	0.002	0.001	0.0005	0.0001		Antibody	
												Loss %	Gain %
1	Raw Heated	Water bath	4 4	4 4	4 1	2 1	1 —	— —	— —	— —	15 10	33	
	Raw Heated	Ice- box	4 4	4 4	4 4	3 4	— 2	— 1	— —	— —	15 19		
2	Raw Heated	Water bath	4 4	4 3	2 1	1 —	— —	— —	— —	— —	11 8	28	
	Raw Heated	Ice- box	4 4	4 4	2 3	2 2	— 1	— —	— —	— —	12 14		
3	Raw Heated	Water bath	4 3	4 2	1 —	— —	— —	— —	— —	— —	9 5	45	
	Raw Heated	Ice- box	4 4	4 4	1 —	— —	— —	— —	— —	— —	9 8		
4	Raw Heated	Water bath	4 3	4 1	2 —	— —	— —	— —	— —	— —	10 4	60	
	Raw Heated	Ice- box	4 4	4 4	4 4	1 4	— —	— —	— —	— —	13 16		
5	Raw Heated	Water bath	4 4	4 4	4 1	2 1	1 —	— —	— —	— —	15 10	33	
	Raw Heated	Ice- box	4 4	4 4	3 4	1 2	— 1	— —	— —	— —	12 15		
6	Raw Heated	Water bath	4 3	4 1	1 —	1 —	1 —	— —	— —	— —	11 4	64	
	Raw Heated	Ice- box	4 4	4 4	1 1	1 1	— —	— —	— —	— —	10 10		
7	Raw Heated	Water bath	4 3	4 2	1 —	— —	— —	— —	— —	— —	9 5	45	
	Raw Heated	Ice- box	4 4	3 3	1 1	— —	— —	— —	— —	— —	8 8		
8	Raw Heated	Water bath	2 2	2 1	— —	— —	— —	— —	— —	— —	4 3	25	
	Raw Heated	Ice- box	2 4	1 4	— 1	— —	— —	— —	— —	— —	3 9		
9	Raw Heated	Water bath	4 1	4 —	— —	— —	— —	— —	— —	— —	9 1	89	
	Raw Heated	Ice- box	4 4	4 3	1 1	1 —	— —	— —	— —	— —	10 8		
10	Raw Heated	Water bath	4 4	4 4	4 3	4 2	3 —	1 —	— —	— —	20 13	35	
	Raw Heated	Ice- box	4 4	4 4	4 4	4 2	2 —	— —	— —	— —	18 14		

amount of antigen employed in the test. It thus appears that these serums belong to a class by themselves and are not representative of the behavior of moderately and weakly reacting serums.

Turning to the effect of inactivation on serums of weak and moderate potency we noted again the phenomenon already alluded to in our previous communication, namely, that the mode of fixation plays a marked rôle in the final results. Thus, after a fixation period of 1 hour in the water bath there was, in most cases, what appeared to be a marked antibody destruction due to inactivation. After a fixation period of 4 hours in the icebox, however, there was usually found, either some gain or a comparatively small loss in antibody content. Tables 1 and 2 illustrate this point.

The experiments in table 1 were carried out with an alcoholic antigen of beef heart and those in table 2 with a cholesterinized antigen. Due to the large amount of tabular material, the effect of inactivation on 10 serums only are recorded in each case. It will be seen that antibody loss is occasionally observed after inactivation even when fixation is carried out in the cold. This loss, however, is comparatively small, while the gain in antibody content is, as a whole, quite pronounced.

DISCUSSION

When employing an alcoholic and cholesterinized beef heart antigen, it was observed that inactivation of serum for one-half hour at 56 C., showed a tendency for stronger complement-fixation reactions compared with raw serums in many cases. This finding is not entirely in disagreement with those of the numerous workers who have shown that inactivation of syphilitic serum has a marked destructive effect on complement-fixing substances. The experiments which led these workers to this conclusion are based on complement-fixation tests carried out with a comparatively short period of fixation. To our knowledge, this period extended from one half to 1 hour in the water bath, in every case. Our findings with a 1-hour period of fixation in the water bath also show marked antibody destruction following inactivation. Our disagreement with these workers is limited to their interpretation of the results. They appear to have assumed that their findings with short fixation periods in the water bath is applicable to prolonged fixation periods in the icebox. This, our studies indicate, is not entirely true. A 4-hour fixation period in the icebox not only shows little antibody destruction due to inactivation, but shows a considerable gain in antibody content in many cases.

Whether the gain in antibody content is due to the colder temperature of fixation or the prolonged period (4 hours) has been considered in our previous paper.⁴ The results indicated that the gain was largely due to the prolonged period rather than to the temperature of fixation.

It would appear that in syphilis we are dealing not with antibody destruction following inactivation, but probably with molecular rearrangement. This rearrangement has a tendency to decrease the velocity of fixation. After a 1-hour period of fixation, the reaction is quite weak; after sufficient time, however, such as 4 hours, the complement-fixation power of the serum is brought forth again. This does not explain why, in many cases, there is an antibody gain following inactivation. Probably the same factors which enhance the precipitation reaction because of inactivation play a rôle in increasing the complement-fixation power.

CONCLUSIONS

The inactivation of syphilitic serum for one-half hour at 56 C. was found to enhance the complement-fixation reaction in many cases, providing the period of fixation was no less than 4 hours at icebox temperature. A small loss in antibody content following inactivation was observed in some cases. The proportional antibody gain, however, due to inactivation, was greater than the antibody loss.

THE RELIABILITY OF THE SACHS-GEORGI TEST FOR SYPHILIS

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As early as 1907 Michaelis,¹ Levaditi and Yamanouchi² and several other investigators expressed the opinion that a precipitate was formed in a positive Wassermann test. Jacobsthal³ was able to show, with the aid of the ultramicroscope, that such a precipitate could actually be observed when a mixture of Wassermann antigen and syphilitic serum were incubated for half an hour at 37 C. Many workers in the years following tried to devise practical methods of visible precipitation which would be specific and reliable in the diagnosis of syphilis. Lang,⁴ in 1912, published the colloidal gold test which has proved to be a helpful method in the diagnosis of cerebrospinal syphilis. The method of Sachs and Georgi⁵ was published in 1918, and soon after this these workers were able to summarize results of 12,124 tests made according to their method in their own laboratory and in those of other workers following their directions. In this series there was an agreement of 92.44% with parallel Wassermann reactions. Because of the simplicity of this test, numerous workers have investigated its reliability. The agreement in the results obtained with parallel Wassermann tests, has been so encouraging that the Sachs-Georgi test is receiving more and more serious consideration as a substitute for the Wassermann reaction in situations in which it is difficult to obtain proper conditions for the successful handling of the more complicated procedure.

Parker and Haigh⁶ of this laboratory have published the results of 520 Sachs-Georgi tests made parallel with the Wassermann test, showing an agreement of 93.07%. The methods were essentially those originally devised by Sachs and Georgi. Since then, D'Aunoy⁷ reported

Received for publication, July 19, 1922.

¹ Berl. klin. Wchnschr., 1907, 44, p. 1477.

² Compt. rend. Soc. d. biol., 1907, 59, p. 740.

³ München. med. Wchnschr., 1905, 56, p. 2607.

⁴ Berl. klin. Wchnschr., 1912, 49, p. 897.

⁵ Med. Klin., 1918, 14, p. 805.

⁶ Arch. Dermat. & Syph., 1921, 4, p. 67.

⁷ Jour. Med. Research, 1921, 42, p. 339.

an agreement of 98.07% in 2,150 hospitalized cases. Taniguchi,⁸ in England, reported an agreement of 91.2% in 296 cases. In general, the greatest divergence of results occurred in weak positive and doubtful reactions. Levinson and Peterson⁹ reported agreement of 78% on serums and spinal fluids in 100 cases of tabes, paresis, cerebrospinal syphilis, etc. In tests carried out on many serums from syphilitic patients in different stages of the disease, Stern¹⁰ found that the Sachs-Georgi test agreed with the Wassermann reaction in 88% of the cases. In spinal fluids there was agreement of 86% in the 2 tests. It was observed in this series that in primary and secondary syphilis the Wassermann test was stronger than the Sachs-Georgi, whereas in latent or treated cases of syphilis the reverse was true. Tannenberg¹¹ cited the collected reports of Pesch from 30 investigators who had compared the Wassermann and Sachs-Georgi tests in 31,000 cases in which there was an agreement of 86.9% of the cases tested. Summarizing from the results obtained by 44 investigators, involving 56,586 cases, it appears that the Sachs-Georgi test agree with the Wassermann test in 88.7% of the tests.

THE REACTION

To obtain the results here reported we have used the technic described by Parker and Haigh, introducing a few minor modifications which expedited setting up the tests and assured the production of an antigen of the proper sensitiveness with somewhat more regularity.

Antigen Extract.—The antigen was prepared from fresh beef heart, trimmed free of fat and connective tissue, and ground fine by passing several times through a meat chopper equipped with the "peanut butter" grinding head. The chopped heart was then mixed with an equal bulk of clean sand, which had previously been thoroughly washed and ignited to remove salts and organic matter, and was ground in a mortar until the mass had the consistency of stiff putty. It was then placed in a ground-glass stoppered bottle containing a few glass beads. Five volumes of absolute alcohol were added and the extraction was effected by shaking mechanically for 5 hours, and then allowing to stand at room temperature for 24 hours. The mass was then filtered through filter paper and placed in the icebox for 48 hours to allow the precipitation of phosphates. These precipitates were removed by filtration

⁸ Brit. Jour. Exper. Path., 1921, 2, p. 41.

⁹ Jour. Nerv. & Ment. Dis., 1921, 54, p. 413.

¹⁰ Ztschr. f. Immunitätsf. u. exper. Therap., 1921, 32, p. 167.

¹¹ Ibid., p. 381.

while the mass was still cold, and the clear antigen extract was placed in tightly stoppered bottles and kept at room temperature.

Titration of the Antigen.—The most important part of the technic in this test is the titration of the antigen, a procedure in which we followed with almost no modification the details as described by Sachs and Georgi and somewhat modified by Parker and Haigh. For specific directions, therefore, we may refer the readers to the last paper. In principle, the procedure consisted in making 3 mixtures of the crude antigen in which 1, 2 and 3 volumes of absolute alcohol were added to 1 volume of the antigen, respectively. Each of these antigen alcohol dilutions, then, was divided into four parts, and to these was added a 1% solution of cholesterol in absolute alcohol sufficient in amount to bring the several fractions to 0.03, 0.045, 0.06 and 0.075% of the cholesterol solution. These cholesterinized antigen dilutions were then diluted with 5 volumes of salt solution. We thus obtained 12 different antigen mixtures, or 3 sets of 4 each, each set containing 1 tube each of equivalent cholesterol concentrations on a varying antigen-alcohol mixture basis. Titration against known positive and negative serums were then made with these in the usual manner and after the first few titrations, controls made with the same serums and an antigen used successfully in previous tests. In general, our optimum antigen dilution was one in which 1 volume of antigen extract had been mixed with 5 volumes of salt solution as described. The actual steps in the method of adding the antigen to the tests is described in detail further on. Two antigens of about equal sensitiveness were used for all the tests made.

Considerable difficulty was experienced in producing good antigens by extracting with 95% alcohol. Absolute alcohol was tried and satisfactory antigens were produced which were reliable. It was found, too, that a 1% solution of cholesterol would remain in solution at room temperature in absolute alcohol, whereas the cholesterol would crystallize out of the 1% solution in 95% alcohol, which necessitated gentle heating to produce complete solution before using each time.

Patient's Serum.—The patient's serum should be clear, fresh and inactivated by heating for one half hour at 56 C. The serum should be allowed to stand 3 or 4 hours after inactivation before the tests are made. Serums used too soon after inactivation were found by Munster¹² to give nonspecific and doubtful reactions. Considerable

¹² München. med. Wehnschr., 1919, 66, p. 505.

amounts of free hemoglobin did not interfere with the test. Cloudiness of a serum made it difficult to read the reaction.

Salt Solution.—Sodium chloride solution, 0.85%, was made in distilled water. A volumetric flask was used to insure uniformity of concentration. The solution was freshly prepared for each set of tests and was sterilized in the Arnold for 1 hour to reduce bacterial contamination of the tests to a minimum. The salt solution was cooled to room temperature before use.

Serum.—Three tubes, 1 for each antigen and 1 for the serum control, were set up for each test. To each tube was added 0.1 c c of the patient's serum and 1 c c of salt solution. To expedite pipetting, we used 1 c c of salt solution and not 0.9 c c, as indicated in the original test, with no appreciable change in the sharpness of the reactions.

Antigen Dilution.—For each test 0.5 c c of the antigen dilution determined as suitable by the preliminary titration was used. Just before setting up the tests, this antigen dilution was prepared in bulk by calculating the total amount needed for all the tests to be done. The calculated amount of the antigen extract was then placed in a clean, dry, 200 c c Erlenmeyer flask and the proper amount of the alcoholic solution of the cholesterol added. An equal volume of salt solution was then rapidly run into the flask from a buret, and the flask was gently rotated to insure complete mixture of the ingredients. A time interval of 10 minutes was allowed to elapse and then in a similar manner 4 more volumes of salt solution were added and the flask gently rotated as before. This method of dilution insured a uniformity in the antigen concentration. By adding the salt solution too rapidly an antigen dilution of slight opalescence and correspondingly slight sensitiveness was produced. When the solution was added too slowly, the extract dilution was very turbid and was found to be either hypersensitive or subject to spontaneous flocculation.

Serum Control.—To one tube of the set was added 0.5 c c of a solution of absolute alcohol, 1 volume to 5 volumes of saline solution—in other words, an amount of alcohol equivalent to the alcoholic content of the antigen used in the tests. A definite flocculation in this tube would invalidate the test. However, in 1,000 tests we did not obtain such a flocculation in the serum control tube. We found the chief value of the serum control tube to be the elimination, by comparison, of doubtful positives in tests in which there was a considerable amount of bacterial growth.

Antigen Control.—An antigen control was used for each antigen in a series of tests. To 1 c.c. of salt solution was added 0.5 c.c. of the antigen dilution. The control tube should remain unchanged throughout the entire incubation period.

Incubation.—Each tube was shaken individually. All tests were then incubated for 20 hours at 37 C. Provisional readings were made at the end of 20 hours, after which the incubation was continued for 20 hours in a cold room at 14 to 16 C., or in the icebox.

Readings.—A test which showed complete precipitation, or with occasional flocculi in suspension and no opalescence in the supernatant liquid, was rated “++++.”

Tests which showed considerable sedimentation with many fine flocculi in suspension were read as “+++.”

Tests which showed a moderate amount of sedimentation with the supernatant liquid filled with very fine flocculi were read as “++.”

TABLE 1
GROSS COMPARISON OF RESULTS WITH THE WASSERMANN AND THE SACHS-GEORGI TESTS

Agreement		Disagreement	
Negative tests.....	676	Wassermann positive.....	} 45
		Sachs-Georgi negative.....	
Positive tests.....	268	Wassermann negative.....	} 7
		Sachs-Georgi positive.....	
Total tests.....	1060		
Agreement.....	94.4%	Wassermann anticomplementary.....	} 4
		Sachs-Georgi, definite results.....	

Tests which showed a slight fine sediment with a turbid supernatant liquid were rated as “+.” Tests in this class frequently must be centrifugalized at slow speed for a short time to determine whether they are positive or not. On shaking a centrifugalized test, if rated as a “+,” a few well organized white flocculi will be suspended. A centrifugalized test containing considerable bacterial growth will contain a sediment which is gray and finely granular, but which can be entirely dispersed in the supernatant liquid on shaking the tube. All of our tests were read without difficulty with the naked eye.

The Wassermann Technic.—The Wassermann tests which were run in parallel with the Sachs-Georgi tests were performed by the serologist, Miss Rockstraw, in the laboratory of Dr. J. G. Hopkins. Two alcoholic

antigens, and one cholesterol antigen containing 0.2% of cholesterol were used in each test. The alcoholic antigen tests were incubated for 4 hours in the icebox prior to the addition of the hemolytic system. The cholesterolized antigen tests were incubated for one hour in the water bath at $37\frac{1}{2}$ C. before the addition of the hemolytic system. The one-tenth Wassermann is used entirely in this laboratory.

Very early in our work we observed minor variations in the results obtained by the Wassermann and the Sachs-Georgi tests. These variations were most prominent in the weak positive serums, and occurred most frequently in old cases and in patients receiving antisyphilitic treatment. An effort was then made to obtain information on the number of treated cases and to ascertain whether a relationship really existed between treatment of the patient and irregularities of findings by the Wassermann and Sachs-Georgi tests. Likewise, considerable variation existed between the cholesterol and alcoholic antigens in the Wassermann tests, which raised the question as to which of these antigens the Sachs-Georgi results most nearly paralleled. To show these findings in a brief and comprehensive manner, the following tables were devised:

TABLE 2
COMPARISON OF THE WASSERMANN AND SACHS-GEORGI TESTS IN DETAIL

Number	Wassermann Tests		Detailed Comparative Readings of the S.-G. Tests					Percentage Agreement as to Positive or Negative Nature of Result
	Cholesterol Antigen	Alcoholic Antigen	++++	+++	++	+	—	
126	++++	++++	92	24	4	3	3	97.6
10	Cholesterol > Alcoholic							
21	++++ or +++	+++ or ++	5	2	1	1	1	90.0
21	++++ or +++	+ or —	5	3	7	5	1	95.2
	Cholesterol < Alcoholic							
58	+++ or ++	++++ or +++	10	16	16	13	3	94.8
27	+ or —	++++ or +++	1	0	3	14	9	59.3
12	+++	+++	2	3	3	3	1	91.7
5	++	++	0	0	4	1	0	100.0
	Cholesterol > Alcoholic							
16	++ or +	+ or —	0	1	1	12	2	87.5
	Cholesterol < Alcoholic							
33	+ or —	++ or +	0	0	3	7	23	30.3
5	—	—	0	0	0	3	2	60.0
683	—	—	0	0	2	5	676	98.9
4	(Anticomplementary)		2	0	0	0	2	—
1000		117	49	44	67	723	

It should be noted that in the 4 cases which were anticomplementary to the Wassermann test a definite diagnosis was obtained by the Sachs-Georgi test in each case. In order to determine the reliability of the Sachs-Georgi findings in these cases the histories of the cases were

obtained. One of the cases which was anticomplementary to the Wassermann and negative to the Sachs-Georgi test was a treated case which had evidently "cleared up" under treatment. Another case which was likewise negative in the Sachs-Georgi test and anticomplementary in the Wassermann test presented an absolutely negative syphilitic history. Of the 2 cases which were anticomplementary in the Wassermann tests and + + + + in the Sachs-Georgi tests, one was that of a tertiary syphilitic. In a subsequent Wassermann test 0.01 c c of this patient's serum in a 1 : 5 dilution was + + + +. The other was a new case in the gynecologic department of the Vanderbilt Clinic in which the patient showed, on examination, venereal warts of the vagina.

TABLE 3
COMPARISON OF THE WASSERMANN AND SACHS-GEORGI TESTS IN TREATED CASES

Number of Treated Cases	Wassermann Tests		Detailed Comparative Readings of the S.-G. Tests					Percentage Agreement as to Positive or Negative Nature of Result
	Cholesterol Antigen	Alcoholic Antigen	++++	+++	++	+	-	
28	++++	++++	16	7	3	1	1	96.4
4	Cholesterol > Alcoholic		2	0	1	0	1	75.0
12	++++ or +++	+++ or ++	3	1	4	4	0	100.0
	Cholesterol < Alcoholic	+ or -						
28	+++ or ++	++++ or +++	5	6	8	7	2	92.8
16	+ or -	++++ or +++	1	0	1	7	7	56.2
8	+++	+++	1	3	2	1	1	87.5
4	++	++	0	0	3	1	0	100.0
	Cholesterol > Alcoholic							
11	++ or +	+ or -	0	0	0	10	1	90.9
	Cholesterol < Alcoholic							
21	+ or -	++ or +	0	0	2	4	15	28.6
3	+	+	0	0	0	1	2	33.3
69	-	-	0	0	2	3	64	92.7
204	28	17	26	39	94	

Of the 204 treated cases, 84 would have been rated + + and above with the cholesterol antigen, 11 would have been + to + +, and 109 + or —. Of the same number of cases the alcoholic antigen would have rated 88 + + and above, 21 + to + +, and 95 + or —. The Sachs-Georgi test would rate 71 of the same number of treated cases + + and above, and 133 + or —.

An analysis of table 3 reveals the following facts:

(1) The Sachs-Georgi test was more sensitive than the Wassermann test in 17 treated cases. Of these cases, 12 or 70.5% were strong positives by both tests.

(2) The Wassermann reaction was more sensitive than the Sachs-Georgi test in 98 treated cases. Sixty-one of these cases or 62.2%,

were strong positives to one or both Wassermann antigens. These figures would indicate that the Wassermann test is more sensitive than the Sachs-Georgi test in weak positive cases under treatment.

(3) The two tests were equally sensitive in 87 treated cases. Of these tests of equal sensitiveness to both methods of diagnosis, 16 were + + + +, 7 were weak positives, and 64 were negative.

In a comparison of the percentage of agreement in the two tests in all cases and in treated cases, all groups of tests, as presented in tables 2 and 3, compared favorably except in the Wassermann group in which cholesterol antigen gave a + + + + or + + + reaction, and the alcoholic antigen a + + + or + +, the agreement being 90% for all cases in the group and 75% for the treated cases in the same group. Also, in the + Wassermann group to both antigens, the percentage of agreement is 60 for all cases and 33.3 for treated cases in the same group. With only 3 exceptions, the percentages of agreement for all cases in the various groups are from 1 to 4 points higher than the corresponding percentages of the treated cases. Of the 3 exceptions, the group in which the Wassermann reaction is + + to both antigens, the agreement is 100% in both tables. In the remaining 2 groups the percentages for all cases are 3 or 4 points lower than the corresponding percentages for treated cases. From a comparison such as the one just presented, it is concluded that the Sachs-Georgi test is not as sensitive as the Wassermann test in cases of patients undergoing antisyphilitic treatment. This idea is supported by the fact that 30 patients under treatment cleared up sooner to the Sachs-Georgi test than they did to the Wassermann test, whereas only 5 treated patients gave a positive Sachs-Georgi reaction when the Wassermann test was negative.

Referring again to table 2, it will be noted that in the strong positive groups in which the cholesterol and alcoholic antigen of the Wassermann did not agree, the Sachs-Georgi results agreed more closely with the cholesterolized Wassermann antigen than with the alcoholic antigen, there being 73 cases in which the Sachs-Georgi test paralleled closely the results obtained by the cholesterol Wassermann antigen, and 43 cases in which the reverse was true. The question could be raised as to why the cholesterol "fortified" antigen should give a weaker reaction than the alcoholic antigen. (For it will be noted in table 2 that in 85 cases the alcoholic antigens in the Wassermann test gave a stronger reaction than the cholesterol antigen, while 31 cases gave reactions in the reverse order.) It is well known that a cholesterol antigen is

supersensitive when subjected to the icebox treatment. Cholesterol being one of the higher alcohols ($C_{26}H_{43}OH$) and fat-like in character, it is possible that in the icebox treatment, cholesterol aggregates are formed which are of such a size and nature as to be able to fix complement mechanically in a manner comparable to the ultramicroscopic precipitate in the positive Wassermann test. Cholesterol is found in appreciable quantities in the bile, and a patient who is troubled with icterus would have some cholesterol in the blood serum which when subjected to the icebox treatment would act in a manner similar to a cholesterol "fortified" antigen and thus give a degree of complement fixation which would seem to indicate that an alcoholic antigen is more sensitive than a cholesterol antigen.

Several cases were encountered in this study which resulted in a total disagreement between the Wassermann and Sachs-Georgi findings. In order to determine which method of diagnosis was the more reliable, histories of the cases were obtained. Since no general classification is possible, these cases are presented in brief form in tables 4 and 5.

In addition to the cases of total disagreement listed, there were 28 cases of partial disagreement, such as a negative result with the cholesterol Wassermann antigen and a +, ++ or +++ results with the alcoholic antigens, in which the parallel Sachs-Georgi tests were negative. Twenty-one of these patients gave a positive syphilitic history or were under treatment at the time of the tests. The remaining 7 patients presented doubtful histories. One case, in particular, gave a history of severe headaches. A lesion was denied by the patient. A Wassermann test made on April 4 was: cholesterol antigen negative, alcoholic antigens + + + +, Sachs-Georgi test negative. Subsequent Wassermann tests made on April 7 and 11 were, respectively, cholesterol antigen negative, alcoholic antigens + +, and negative for all antigens. Another case of an opposite character likewise gave no history of an initial lesion. The patient had been treated for 4 years for tuberculosis of the throat. At the time of the test the patient complained of rheumatic pains and had on the chin a serpiginous crypt irregular in shape with a scaly crusted center. The Wassermann test was negative with the cholesterol antigen and + with the alcoholic antigen. The parallel Sachs-Georgi test was negative. A subsequent Wassermann test was + + + +.

Three cases were encountered which gave a ++ or +++ reaction with the cholesterol Wassermann antigen and negative results with the alcoholic antigens, while the parallel Sachs-Georgi tests were nega-

tive. Two of these patents gave positive syphilitic histories. The remaining patient gave no definite history of syphilis. The serum of this patient was unfit for any test, being very turbid and contained a great amount of released hemoglobin. In the entire group in which the Wassermann test was positive in the cholesterol antigen tests and

TABLE 4
CASES OF DISAGREEMENT BETWEEN THE WASSERMANN AND SACHS-GEORGI TESTS
(SACHS-GEORGI NEGATIVE)

Patient	Wassermann			Sachs-Georgi	History
	Cholesterol Antigen	Alcoholic Antigen	Alcoholic Antigen		
J. V.	++++	++++	++++	—	Lesion denied. Paralysis of left recurrent laryngeal nerve, partial paralysis of right recurrent laryngeal nerve. One knee jerk absent. Under antisyphilitic treatment
T. S.	++++	++++	++++	—	Contracted syphilis 9 years ago. Just prior to present test developed osteomyelitis as a result of an injury in an automobile accident
A. E.	++++	++++	++++	—	Developed a chancre 7 years ago. Received 2 antisyphilitic treatments at that time. At present complains of headache and malaise. Pupils sluggish
Harris..... (Baby of V.)	++	++++	++++	—	Cord Wassermann. Mother was receiving antisyphilitic treatment at the time of the present tests
M. G.	+	++++	++++	—	Tertiary syphilis, under treatment at the time of the test
B. B.	+	++++	++++	—	Children were positive to the Wassermann test. Received antisyphilitic treatment prior to test
S. C.	+	++++	++++	—	A new case in the Vanderbilt Clinic; migraine. Symptoms began 2 years ago with gradual onset. Clinical diagnosis of syphilis doubtful
M. A.	+++	+++	+++	—	Under antisyphilitic treatment at time of the test
S. C.	++	+++	+++	—	A treated case. Treatment was discontinued 2 months prior to test
V. B.	+++	++	++	—	Previously diagnosed as syphilis. Under treatment at time of the test
M. S.	+	+	++	—	Gonorrheal infection 10 years ago. Complaints of pain in the feet. Diagnosed subcutaneous syphiloma. A subsequent Wassermann test was ++++ for all antigens

* Most of the cases in this group represent old cases, congenital syphilis and treated cases which "cleared up" early to the Sachs-Georgi test. The Sachs-Georgi test appears to be unreliable when used with cases of this nature.

negative to the alcoholic antigens, the Sachs-Georgi tests were positive in 18 cases and negative in 3 cases. Of the 18 cases of agreement of the Wassermann cholesterol antigen and the Sachs-Georgi test, 11 were treated cases and 3 cases presented positive syphilitic histories.

In the two groups just discussed, composed of weak positive (not agreeing in the results obtained with the cholesterol and the alcoholic

antigens) according to the Wassermann test, the Sachs-Georgi tests gave results which agreed exactly with the results obtained with the cholesterol Wassermann antigen in 46 of the 49 cases tested.

SUMMARY

When roughly classified into "positive" and "negative," the Sachs-Georgi tests agreed with the Wassermann tests in 94.4% of 1,000 cases tested.

The Sachs-Georgi test appears to be less sensitive than the Wassermann reaction in patients receiving antisyphilitic treatment and in cases of cerebrospinal syphilis.

The Sachs-Georgi test is just as sensitive as the Wassermann test in untreated cases of primary, secondary and tertiary syphilis.

TABLE 5
CASES OF DISAGREEMENT BETWEEN THE WASSERMANN AND SACHS-GEORGI TESTS
(SACHS-GEORGI POSITIVE)

Patient	Wassermann (All Antigens)	Sachs-Georgi	History
A. T.	—	++	A treated case. Treatment was discontinued 6 weeks prior to test
M. P.	—	++	Receiving antisyphilitic treatment at time of the test
F. S.	—	+	Antisyphilitic treatment discontinued 3 weeks
T. McG.	—	+	Chancere on the penis 20 years ago. Chronic eczema on left shin. Tertiary syphilis
C. M.	—	+	Fibroid (no record of any kind could be obtained of this case)
L. S.	—	+	Tertiary syphilis; under treatment at time of test
A. W.	—	+	Antisyphilitic treatments were discontinued 4 months prior to test

All of these cases, except T. McG. and C. M., may be interpreted as treated cases which "cleared up" to the Wassermann test earlier than to the Sachs-Georgi.

The specificity of the Wassermann alcoholic antigen under the four-hour icebox treatment can be questioned when the presence of bile salts in the patient's serum is ignored. With this unknown quantity controlled, the Sachs-Georgi test would show a still more favorable comparison with the Wassermann reaction as a routine test.

In four cases in which the patient's serum was anticomplementary in the Wassermann test, the Sachs-Georgi test gave a definite and reliable diagnosis in each case.

Because of the simplicity and reliability of the test, as shown by the data herein presented, the Sachs-Georgi test should attain a range of usefulness in the diagnosis of syphilis far in excess of that accredited to it at the present time.

IMMUNOLOGIC EXPERIMENTS WITH PLATELETS OF HUMAN BLOOD

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Marino¹ produced an antiserum in rabbits with platelets from the guinea-pig and found that this antiserum destroyed guinea-pig platelets, but was not toxic to the guinea-pig when injected intravenously. Le Sourd and Pagniez,² repeating the experiments of Marino, studied also the effect of antiplatelet serum on the clotting time of the corresponding blood, and concluded that the antiserum was lytic for platelets but caused no delay in the clotting time and was not toxic for guinea-pigs. These results were confirmed also by Chevel and Rogers.³ Cole⁴ separated the platelets from human blood, injected rabbits and demonstrated agglutination of the platelets with the rabbit antiserum, which had no effect on the clotting time of human blood. Sacerdotti⁵ obtained an antirabbit platelet serum from the dog, and demonstrated its specificity and found it highly toxic in vivo. Injection of rabbits with the antiserum caused platelet reduction, hemorrhages and death in shock. Repeating this experiment, Stschastnyi⁶ was unable to confirm the specificity of the serum. Aynaud⁷ also disagreed with Sacerdotti and concluded that the antiserum was species specific but not cell specific, agglutinating and causing lysis of platelets in vitro, properties shared also by antiserum for red corpuscles. He was unable to demonstrate a specific effect of antiplatelet serum by absorption tests.

Ledingham and Aberd,⁸ studying the relationship of antiplatelet serum to the so-called hemorrhagic diathesis in man, confirmed the power of platelet antiserum to bind complement and were unable to demonstrate this property in anti-erythrocytic serum. They obtained other results like those mentioned. The effects of subcutaneous and

Received for publication, July 21, 1922.

¹ Compt. rend. Soc. de biol., 1905, 58, p. 194.

² Compt. rend. Acad. de sc., 1906, 118, p. 562.

³ Compt. rend. Soc. de biol., 1907, 63, p. 501.

⁴ Bull. Johns Hopkins Hospital, 1907, 17, p. 261.

⁵ Arch. ital. biol., 1908, 52, p. 153.

⁶ Folia Serol., 1909, 2, p. 285.

⁷ Compt. rend. Soc. de biol., 1911, 70, p. 54.

⁸ Lancet, 1914, 186, p. 1673.

intravenous injections of antiginea-pig platelet serum in guinea-pigs were striking; subcutaneous hemorrhages, phagocytosis and agglutination of red corpuscles and reduction in platelets. This work was repeated in more detail by Ledingham and Bedson,⁹ who were unable to obtain any such results with antileukocytic serum and concluded that the action was due to a strong lytic and agglutinating power of anti-platelet serum. Lee and Robertson¹⁰ confirmed these observations and pointed out that the action was dependent on complement, noting also that the serum of guinea-pigs with experimental purpura hemorrhagica had no effect on platelets *in vivo* or *in vitro*.

More recently, Bedson¹¹ demonstrated specific agglutination of rabbit antiserum of guinea-pig platelets by absorption tests controlled with antisera for other blood elements. He concluded that the early results of Sacerdotti were correct and maintained that the antiplatelet serum is cell specific and that platelets are independent elements of the blood without genetic relationship with other blood cells. He further¹² showed by absorption tests that antiplatelet serum also can produce hemorrhagic purpura.

With the exception of Cole, none of the workers cited studied human blood elements, and it seemed advisable therefore to investigate the immunologic properties of the platelets of human blood.

PREPARATION OF ANTIGENS

Human platelets were obtained from the blood of patients with hypertension, bled for therapeutic purposes (Drs. Koessler, Bookbinder and Borsack). The usual method of fractional centrifuging was used for their separation. It was found almost impossible to remove all of the erythrocytes and leukocytes because of the strong tendency for compact clumping of the leukocytes and platelets, the lighter erythrocytes remaining in the supernatant platelet suspension. The latter would often appear free from red cells under the microscope, but by high speed centrifugation, a definite red mass of cells would form. The variation in size of platelets also affects their separation by this procedure so that even with great care a more or less impure antigen necessarily results. The platelets obtained as outlined were washed 5 times with salt solution; each cubic centimeter of the final suspension contained the platelets from 30 c c of blood.

⁹ *Ibid.*, 1915, 50, p. 311.

¹⁰ *Jour. Med. Research*, 1916, 38, p. 323.

¹¹ *Jour. Path. & Bacteriol.*, 1921, 24, p. 469.

¹² *Ibid.*, 1922, 25, p. 94.

The leukocytes were obtained from the early exudate of gonorrheal arthritis (Drs. Herrold and Forester). Such exudates may yield perfect suspensions of apparently unchanged leukocytes, largely of the polymorphonuclear variety. The leukocytes were washed 5 times in large volumes of salt solution and then made into 10% suspension for injection. No bacteria could be found in the final suspension, although the whole exudate immediately after removal gave positive gonococcus cultures.

Erythrocytes and serum were obtained in the usual manner. The erythrocytes were washed six times with salt solution and suspended in salt solution.

PRODUCTION OF ANTISERUMS

The antisera were produced as follows: The platelet suspension was injected intravenously in rabbits at 3-day intervals in doses of 1, 2, 4, 6, and 8 c.c. Because of the strong tendency to clumping, the leukocytes were injected intramuscularly also at 3-day intervals in quantities of 1, 2, 3, 4, and 6 c.c. of a 10% suspension. Other rabbits injected intravenously with a 1:20 extract of leukocytes also produced an antileukocytic serum. The erythrocytes were given intravenously on 6 succeeding days in 1 c.c. doses of the solid corpuscles after centrifugation and human serum was given intravenously in 4, 8, 10, and 12 c.c. quantities also at 3-day intervals. The animals were bled on the fifth and tenth days after the last injection.

For precipitin tests platelet extract was made by suspending washed platelets in sterile distilled water in the proportion of 1:10 by weight; after shaking for several hours, an equal quantity of sterile 1.8% salt solution was added so that an extract of about 1:20 in 0.9 salt solution was obtained. The portion of the platelets that actually went into solution was of course not known. The subsequent dilutions of the centrifuged clear extract were made on the basis of the original proportion of platelets in distilled water.

Leukocytic extracts were made in a similar manner, and the subsequent dilutions were made on the basis of the original proportion in distilled water. The erythrocytic extract was made by suspending carefully washed corpuscles of a definite quantity of blood in many times that quantity of sterile distilled water, then adding as much again of 1.8% salt solution and centrifuging thoroughly. In this way, clear solutions with 0.9 sodium chloride were obtained; 50 c.c. of this extract contained the corpuscles from 1.0 c.c. of blood. Extract of the stroma of red corpuscles was prepared by thoroughly washing the corpuscles and laking with about 5 times the quantity of distilled water, to which was added a small amount of ether to insure complete dissolution. To this solution an equal quantity of saturated ammonium sulphate was added. The resulting precipitate was filtered, washed with water and salt solution, ammonium sulphate again added and the washing repeated. The final dilution of the precipitate in 0.9 salt solution was one part of precipitate in 40 parts of salt solution. The clear portion removed after centrifuging was used for the precipitin test.

All precipitin tests were made by overlaying the antiserum with extract or serum and looking for a ring at the point of contact after one hour at room temperature.

To determine the agglutinin titer, the suspension of platelets used was similar to that injected. Dilutions of antisera were added to this suspension. For agglutination test with erythrocytes a 2.5% (5% whole blood) suspension was used. The lytic titer was determined by the use of similar suspensions with the addition of 0.025 c.c. of fresh guinea-pig complement to each tube, the complement unit being determined by titration against a constant quantity—0.1 c.c.—of anti-erythrocytic serum. In both of these tests as well as that for opsonins the antisera were inactivated first at 56 C for half an hour. For opsonic titration progressive dilutions of antiserum were added to mixtures of human corpuscles and dog leukocytes obtained from the pleural cavity 12 hours after an intrathoracic injection of sterile aleuronat. The leukocytes were drawn into a warm 2% citrate solution and washed with warm salt solution being added to the erythrocytic suspension.

Table 1 is an illustrative record of the main results.

TABLE 1
TITRATIONS OF ANTIPLATELET AND OTHER ANTISERUMS

Antigen	Antiserum				Normal Rabbit Serum
	Platelet	Leugo-cytic	Erythro-cytic	Serum	
Precipitin Titers					
Platelet extract.....	200	0	100	0	0
Leukocytic extract.....	0	640	0	0	0
Extract of erythrocytic stroma.....	100	0	800	0	0
Erythrocytic extract.....	0	0	80	200	0
Serum.....	200	0	400	6400	0
Agglutinin Titers					
Platelets.....	1536	24	96	48	0
Erythrocytes.....	384	48	3072	768	0
Lytic Titers					
Platelets.....	24	0	0	0	0
Erythrocytes.....	24	0	1536	96	0
Opsonic Titers					
Human erythrocytes and dog leuko-cytes.....	768	0	1536	3072	0

The figures represent the highest dilutions of the antisera at which the results were obtained in case of agglutination, lysis, and opsonification. In the case of the precipitin tests, the figures represent the highest dilutions of the antigens prepared as described in which a definite precipitate was obtained with the antisera after one hour at room temperature.

In considering the precipitin titers of the various antisera, it must be remembered that the solubility in water of the protein constituents of the blood cells are not known. Kaempler¹³ quotes Fornio as stating that platelets are rich in thrombokinase which is yellow and water soluble, but he does not mention the extent of the solubility and no other information bearing on this point was found. In the light of these facts, the figures in table 1 may indicate a much higher specificity of reaction than they appear to do.

It is apparent that the leukocytic antiserum is strictly specific; the serum antiserum is also quite specific, reacting, however, with erythro-

¹³ Am. Jour. Surg., 1915, 29, p. 401.

cytic extract in a 1:200 dilution, but this may be explained by the presence of a small amount of erythrocytic elements in human serum. The precipitates formed by bringing platelet antiserum in contact with extract of erythrocytic stroma and with human serum in low dilutions may be explained by the presence of some corpuscles in the platelet suspension used as antigen and then the serum undoubtedly contains some platelet elements in solution. Werbitski¹⁴ claims to have extracted rabbit platelets with the serums of man and animals and to have studied the bactericidal properties of such extracts, but no other reference to the serum solubility of platelets has been found.

In order to test further the specificity of the platelet antiserum it was diluted 1:100 and thoroughly mixed with an equal quantity of normal human serum. After standing at room temperature for one hour this mixture was centrifugated and the clear solution tested with platelet extract: A definite precipitate formed at the junction point showing that the serum failed to remove the specific precipitin for platelet extract.

The agglutinin tests of the various antisera also indicate specificity. In order to further substantiate this, 1 part of platelet antiserum was treated with 2 parts of a heavy suspension of platelets, the mixture being incubated at 37 C. for 2 hours and then centrifugated. The antiserum so treated agglutinated platelets in a dilution of 1:24 and erythrocytes in a dilution of 1:192, 1:384 being the titer of the untreated antiserum for erythrocytes. Similarly, platelet antiserum was mixed with 2 parts of a 25% suspension of human erythrocytes (50% human blood) in salt solution for 2 hours at 37 C. The treated antiserum now agglutinated platelets in a dilution of 1:768—about one half the original titer—and erythrocytes in a dilution of 1:1 only. While the platelets were able to remove the agglutinins from the platelet antiserum so that subsequent agglutination was reduced to a minimum, erythrocytes under the same conditions failed to remove the agglutinins for platelets.

The agglutinin titer for human leukocytes could not be determined accurately, because of the marked spontaneous clumping of leukocytes, and this phase of the work is to be taken up again later.¹⁵

¹⁴ Ztschr. f. Hyg. u. Infektionskr., 1911, 68, p. 63.

¹⁵ While this article was in preparation there appeared a report by Rosenthal and Falkenheim on agglutinins for different elements in human and chicken blood in which they conclude that the platelets are related more closely to the leukocytes than to the erythrocytes (Arch. f. exp. Path. u. Pharmacol., 1922, 92, p. 231).

Apparently the antiserums were not rich in lysins, and pronounced lytic effect was obtained with erythrocytes and erythrocytic antiserum only. A prompt clearing occurred of platelet suspension with low dilutions of platelet antiserum and no effect on platelets was noticed with other antiserums. Opinion appears to be divided as to the lytic action of platelet antiserum for platelets. As stated, complement fixation has been demonstrated by several, but my results with complement were not so definite or uniform as those obtained with agglutinin and precipitin tests.

It is interesting to note that phagocytosis of red corpuscles was not observed with antileukocytic serum, while platelet antiserum gave an opsonic titer of 768. The presence of opsonins in the platelet antiserum may be explained as the result of the antigenic effect of erythrocytes in the platelet suspension injected as antigen.

The reactions described are in harmony with the results Bedson obtained with guinea-pig blood. Evidently platelets contain specific antigens, as might be expected from the morphologic observations of Wright ¹⁶ and others as to their origin from megakaryocytes in marrow.

SUMMARY

Immunological reactions of human platelets indicate the presence in platelets of specific antigenic constituents, and results of the precipitin test point to a definite difference in the constitution of the platelets and the leukocytes in human blood. The observations on human platelets support the views of Bedson and others that the platelets differ in their constitution from other elements of the blood.

¹⁶ Boston Med. and Surg. Jour., 1906, 154, p. 643.

THE INTESTINAL BACTERIAL FLORA OF RATS ON A DIET DEFICIENT IN FAT SOLU- BLE VITAMIN A

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Intensive work of recent years appears to have established the specific nature of certain deficiency diseases of lower animals which can be made the subject of experimental investigation. Polyneuritis of fowls, scurvy of guinea-pigs, xerophthalmia and abnormality of calcification of rats and "leg weakness" of chicks are conditions which are apparently associated with the lack of essential and specific dietary constituents. The experimental diets used in such studies deviate quite widely from the normal diets of the various species of animals in a natural environment, and the deficient diet causes not merely the conditions mentioned, but also fails to support the growth of the animal. Although the test diet plus minute quantities of the specific substance or vitamin is adequate for growth, the question may be raised as to whether faulty assimilation due to abnormalities within the intestinal canal may have a part in the causation of the condition finally established.

McCarrison,¹ in his clinical and experimental study of the general metabolic disturbances associated with faulty diets, has ascribed a large share of the resulting condition to definite anatomic and physiologic abnormalities of the gastro-intestinal tract. It must be admitted, however, that the disturbances studied by McCarrison probably do not belong in the same category as the more striking and characteristic vitamin deficiency diseases mentioned. But even in some of the latter there has been some divergence of opinion as to the relative importance of vitamin deficiency and intestinal abnormality.

McCollum and Pitz² have claimed that experimental scurvy in the guinea-pig is not due to any vitamin deficiency but is rather the result of the absorption of toxic substances derived from putrefaction in the cecum caused by undue retention of feces. Any diet which does not possess such physical properties as will lead to the formation of bulky, easily eliminable feces, will produce scurvy in guinea-pigs. The earlier work of Jackson and Moody³ supports this view in so far as intestinal abnormality is concerned, but ascribes greater importance to bacterial infection than to toxin absorption. They described coccus-like bodies in the lesions of scorbutic guinea-pigs and suc-

Received for publication July 26, 1922.

¹ Jour. Am. Med. Assn., 1922, 78, p. 1.

² Jour. Biol. Chem., 1917, 31, p. 229.

³ Jour. Infect. Dis., 1916, 19, p. 511.

ceeded in isolating a gram-positive diplococcus of low virulence which had a tendency to form chains and produced green on blood agar. Pure strains of this organism, when inoculated into the circulation of guinea-pigs living under ordinary conditions, gave rise in most instances to hemorrhagic and other lesions in bones, joints, muscles, lymphnodes and gums, the lesions being similar to those of guinea-pigs in which scurvy developed spontaneously as the result of faulty diets. They also observed that a cream diet and one of olive oil with milk led to fat constipation and early death. The latter finding McCollum and Pitz² believe supports the view that undue retention of feces is the primary factor in the development of scurvy in the guinea-pig.

Funk⁴ and Torrey and Hess,⁵ on the other hand, are of the opinion that scurvy is a deficiency disease due to the absence of the antiscorbutic vitamin. Torrey and Hess⁵ investigated the relation of putrefactive bacterial activity in the intestinal tract of scorbutic guinea-pigs. They made a study of the intestinal flora of guinea-pigs on normal diet, on a diet which produced scurvy, and on a diet which cured this disorder. They found that there was no change in the flora at any time, although the scorbutic symptoms disappeared on a diet which supplied the supposed deficiency.

In the case of "leg weakness" of chicks, there has also been some difference of opinion. Osborne and Mendel⁶ had claimed that in this condition the lack of suitable roughage in the ration is an important factor, the roughage being necessary for the formation of easily eliminable feces. Hart, Halpin and Steenbock⁷ confirmed these findings, but later⁸ found that the addition of cod-liver oil protects chicks against the development of "leg weakness," and that the disease develops if this vitamin-containing accessory is omitted even if suitable roughage is supplied.

That dietary changes may cause alterations in the intestinal bacterial flora has been established by Rettger and his co-workers.⁹ Such alterations are especially easily produced in the rat, the classical test animal in studies of the fat soluble vitamin A. In connection with some other work in which rats were being used for a study of this accessory factor, it seemed important to investigate the changes which might occur in the intestinal flora and in the physical characters of the feces in order that possible intestinal factors might be properly evaluated in the interpretation of the general constitutional changes which occurred in the animals.

White rats were used. The feces were collected according to the manner described by Hull and Rettger.¹⁰ The rats were held by the tail and rubbed on the back at the base of the tail. The feces were collected directly into sterile test-tubes and weighed. They were then

⁴ Biol. Chem. Bull., 1915, 4, p. 304.

⁵ Proc. Soc. Exper. Biol. and Med., 1918, 15, p. 74.

⁶ Jour. Biol. Chem., 1918, 33, p. 433.

⁷ Ibid., 1920, 43, p. 421.

⁸ Ibid., 1922, 52, p. 378.

⁹ A Treatise on the Transformation of the Intestinal Flora, 1921.

¹⁰ Jour. Bacteriol., 1917, 2, p. 47.

emulsified in sterile salt solution in the ratio of 1 gm. of feces to 10 c c of solution. From this primary emulsion, dilutions of 1:1,000, 1:10,000, and 1:100,000 were made and plated. From the original emulsion smears were prepared for staining and microscopic examination. The water content of the diet varied so little that this factor was believed to be without effect on the bacterial counts.

The rats were fed on a diet known to cause vitamin A deficiency diseases. This consisted of casein 18%, dextrin 72%, salts 4% and yeast 6%. In addition, the control rats received from 2 to 3 drops of cod-liver oil. This is considered, and was found to be, an adequate normal diet in regard to vitamin requirement. Whenever the rats on the deficient diet became ill, they were given from 2 to 4 drops of a cottonseed oil solution of an unsaponifiable residue from cod-liver oil which was partly freed from cholesterol. This immediately caused the disappearance of those manifestations which had been brought about by the deficient diet. The fecal material of the control rats, of the rats on a diet deficient in vitamin-A, and of the treated rats on the diet with the unsaponifiable residue was used for comparative study.

The following mediums were used, being inoculated at 37 C. and colony counts made at the end of 48 hours:

Beef liver glucose agar, with an initial reaction of PH 6.8. On this the characteristic fluffy colonies of *B. acidophilus* were produced.

Endo's agar was used for the determination of the proportion of bacteria able to ferment lactose; these were considered as being probably *B. coli*.

Glucose and saccharose agar plates with Andrade indicator permitted the estimation of the number of bacteria which fermented these two sugars.

Blood agar plates were used for counting streptococcus colonies.

Lead acetate agar gave quantitative information as to the number of bacteria able to form hydrogen sulphide.

Gram stains were made of smears of the fecal emulsions and a differential count was made of the gram-positive rods, gram-negative rods, gram-positive cocci and gram-negative cocci. The successive counts on the individual rats were averaged, and from these average counts for all the rats on the control diet and for those on the deficient diet an average for each group of rats was obtained.

On the control diet the percentage of gram-positive rods varied from 11.8 to 19.6 in the individual animals, with a group average of 16.8; in the animals on deficient diet the percentage varied from 11.3

to 24.8, with an average of 17.4. In the control group the percentage of gram-negative rods varied from 66.8 to 72.7, with an average of 69.8; in the experimental group from 62.3 to 75.3, with an average of 68.9. Gram-positive cocci varied from 7.5 to 15.5% in the control animals, with an average of 12.1%, and from 7.7 to 17.8% in the rats on deficient diet, the average being 11.3%. Gram-negative cocci varied from 0.5 to 2.7% in the control group, with an average of 1.3%; in the other group the individual variations were from 0.8 to 3.7%, the average being 2.4% (table 1). The deficient diet, therefore, caused no noteworthy changes in the proportion of the 4 groups of bacteria based on gram stain and morphology. Streptococci disappeared entirely from the flora of 7 of the rats on low vitamin diet. In 4 of these ani-

TABLE 1
DIFFERENTIAL FECAL BACTERIAL COUNTS OF RATS ON NORMAL DIET AND ON DIET LACKING VITAMIN-A

Normal Diet					Diet Deficient in Vitamin-A				
Rat No.	Gram-Positive Rods %	Gram-Negative Rods %	Gram-Positive Cocci %	Gram-Negative Cocci %	Rat No.	Gram-Positive Rods %	Gram-Negative Rods %	Gram-Positive Cocci %	Gram-Negative Cocci %
102	19.6	66.8	13.2	0.5	106	11.3	75.3	12.7	0.8
103	19.0	72.7	7.5	0.8	110	13.4	74.5	10.2	2.0
104	11.8	70.0	15.5	2.7	112	18.3	68.2	10.7	2.9
					201	18.1	72.3	7.7	1.9
					204	24.8	62.7	8.8	3.7
					205	17.1	62.3	17.8	2.8
					206	18.7	67.3	11.2	2.8
Average....	16.8	69.8	12.1	1.3		17.4	68.9	11.3	2.4

imals the *B. acidophilus* percentage went up. In the other 3 the percentage of gram-negative rods rose, or both gram-negative and gram-positive rods increased proportionately. There was no change in the differential count when the vitamin-containing residue was added to the diet, although all symptoms of sickness disappeared from the rats when this addition was made.

The bacterial count per cubic centimeter of the original emulsions differed to a marked degree as the rats were kept on the diet lacking in fat soluble A. The feces became dry and hard as the disease became more advanced. With this change in the physical character of the feces, the bacterial count decreased. Within 24 to 48 hours after the unsaponifiable residue had been given, the feces became moist and soft and the bacterial count greatly increased (table 2). Whether this change, which was striking and the only one noted, was

due to an absence from the intestinal canal of accessory factors perhaps necessary for bacterial growth, to the low food intake of the animals in their ill condition, or to a nutritional condition of the host animal so poor that it could not support even a normal intestinal flora, is left undecided. That it is a fundamental factor in the causation of the condition characteristic of vitamin A deficiency is doubtful. The quantitative alteration in the intestinal flora may be a secondary factor in bringing about the disturbed general metabolism of rats on a deficient diet, by altering digestion within and assimilation from the

TABLE 2
FECAL BACTERIA PER C C BEFORE AND AFTER ADDITION OF VITAMIN-A TO DIET

Rat No.	Date	Glucose Fermenters	Streptococci	B. acidophilus	Remarks
202	4/11	70,000	200,000	230,000	Vitamin-A given May 23, 14, 25 and 26
	5/26	4,000,000	None	100,000	
	5/31	15,000,000	None	100,000	
	6/ 7	2,000,000	None	100,000	
206	4/11	700,000	60,000	160,000	Vitamin-A given June 1, 2, 3, and 5
	5/11	900,000	50,000	140,000	
	5/18	200,000	4,000	39,000	
	6/ 5	1,400,000	None	120,000	
112	4/17	63,000	10,000	10,000	Vitamin-A given May 15, 16, 17, 19, 20, 22, 23, 24, 25, 26, 27, 29, 31, June 1, 2, 3, 5, 6, 9
	4/20	700,000	30,000	15,000	
	5/15*	2,000,000	45,000	8,000	
	5/24	1,000,000	30,000	120,000	
	5/25	4,000,000	200,000	200,000	
	5/26	3,500,000	50,000	200,000	
	6/ 1	4,000,000	20,000	150,000	
201	4/11	160,000	21,000	45,000	Vitamin-A given May 25, 26 and 27
	4/20	350,000	5,000	250,000	
	5/ 5	2,000,000	3,000	320,000	
	5/18	130,000	None	90,000	
105	5/25*	20,000,000	860,000	130,000	Vitamin-A given May 24, 25, 26, 27, 29, 31, June 1, 2, 3, 5, 6, 7, 8, 9
	4/10	200,000	360,000	350,000	
	6/ 5	49,000	None	11,000	
207	6/ 8	60,000	12,000	None	Vitamin-A given May 14, 15 and 16
	4/14	64,000	None	10,000	
	5/18	37,000	None	8,000	

* First dose of vitamin administered at 1 p. m., fecal specimen taken at 3 p. m.
All the rats of this group were placed on deficient diet March 24.

intestinal tract. Even this change in the number of bacteria is not a constant one. In the group of 5 rats included in table 2, the low bacterial count persisted in 105 and 207, even after the addition of vitamin-A, although the evidences of illness disappeared. In the case of rats 112 and 201 (table 2), there was a sudden great increase in the fecal bacterial content on the day that the vitamin was first administered, the increased count being evident in fecal samples taken 2 hours after the administration of the vitamin. No explanation of such a sudden increase is attempted. Whenever the fecal bacteria increased after the administration of vitamin-A the increase was

chiefly of the glucose-fermenting bacteria, streptococci and *B. acidophilus* remaining unchanged or undergoing inconstant changes in number.

The glucose, lactose and saccharose mediums showed no differences associated with the change of diet, except increase or decrease as the total number of bacteria changed.

The ratio of hydrogen sulphide forming bacteria to total bacteria, as determined by lead acetate agar plates, varied from 1:3.4 to 1:13.8 in the control group, the average being 1:8; the total number of sulphide-forming bacteria was fairly constant, but there was considerable difference in the total bacterial counts of the individual animals. In the rats on low vitamin diet, both the hydrogen sulphide formers and the total bacteria were more uniform; the ratio of the former to the latter varied from 1:6 to 1:8.3, average 1:7.2 (table 3). The changes noted are so slight as to be without significance.

TABLE 3
HYDROGEN SULPHIDE FORMING BACTERIA OF RATS ON NORMAL DIET AND ON DIET DEFICIENT IN VITAMIN-A

Normal Diet				Diet Deficient in Vitamin-A			
Rat No.	No. of Sulphide Formers per C c	Total Bacteria per C c	Ratio	Rat No.	No. of Sulphide Formers per C c	Total Bacteria per C c	Ratio
102	12,000	40,750	1: 3.4	106	6,666	44,666	1:6.7
103	7,700	57,000	1: 7.4	110	8,000	71,200	1:8.3
104	10,000	138,500	1:13.8	112	5,714	42,428	1:7.4
				201	5,000	40,800	1:7.3
				206	7,750	46,500	1:6
Average	9,900	78,750	1:8.0		6,866	49,119	1:7.2

SUMMARY

In a study of the fecal bacteria of a group of rats on a diet so deficient in fat-soluble vitamin A as to result in xerophthalmia and other conditions characteristic of this deficiency, as compared with a group on an adequate control diet, no change occurred in the relative proportions of gram-negative rods, gram-positive rods, gram-positive cocci, and gram-negative cocci, as determined by smears made from standard emulsions of the freshly collected feces.

On the deficient diet the feces of the majority of the animals became dry and hard and the total number of viable bacteria was greatly decreased. When these animals were given fat soluble A in an amount sufficient to cause a disappearance of lesions, the feces became moist and soft and the total number of bacteria increased strikingly.

In most of the animals on the deficient diet, streptococci disappeared completely. This may have been due to the unfavorable conditions brought about in the intestinal canal by the inadequate diet, the less resistant streptococci disappearing while the more hardy species merely decreased greatly in number.

There was no change in the proportions of bacteria which fermented glucose, lactose and saccharose.

The proportion of hydrogen sulphide forming bacteria remained constant.

DIRECT INJECTION OF *B. TYPHOSUS* INTO THE GALLBLADDER

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The work of Rous and McMaster¹ following less successful attempts of Rost,² Werelius³ and Mann⁴ indicates that the wall of the gallbladder has a definite part in concentrating bile. This function is so active that when estimates are based on pigment readings, reduction in volume of cystic bile amounts approximately to nine-tenths in 22 hours. These observations have drawn attention to an activity hitherto not well recognized. Meltzer⁵ has indicated that the gallbladder is under nerve control, which leads one to suspect that this function may be under central direction. As the walls of this organ are richly supplied with lymph channels, it seems likely that this intense activity may be related to lymph circulation.

The work cited causes one to consider the possibility of infection or of intoxication to the general system by deleterious bodies or compounds within the cystic bile. The older idea that presence of *B. typhosus* within the gallbladder in a typhoid carrier is due to direct invasion of the bacilli by way of the common and cystic ducts from the duodenum no longer seems tenable. However, there is still strong reason to believe that this may be the path of invasion for *Endameba dysenteriae* and for certain parasites. One may conceive that a foreign toxin-producing body within the bile may be able to produce a generalized condition in the body as the wall of the gallbladder has been demonstrated to comprise an active, permeable and concentrating mechanism.

With this possibility in mind, it was attempted to learn whether the introduction of *B. typhosus* within the gallbladder directly is followed by the appearance of bacilli and of agglutinins in the circulation.

The strain of *B. typhosus* used in these experiments is known as "No. 3" in this laboratory and is the same as that used in a large

Received for publication, Aug. 1, 1922.

* Aided by grant from University of California Board of Research.

¹ Jour. Exper. Med., 1921, 34, pp. 47 and 75.

² Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1913, 26, p. 710.

³ Surg., Gynec. & Obst., 1917, 25, p. 520.

⁴ New Orleans Med. & Surg. Jour., 1918-19, 71, p. 80.

⁵ Am. Jour. Med. Sc., 1917, 153, p. 469.

portion of the work reported in previous communications from this department. It was cultivated on 6% rabbit blood agar according to the method outlined by Gay and Claypole,⁶ that is in tubes of 18 mm. internal diameter with slopes as long as possible without wetting the cotton plugs. Inoculations from a broth culture were made over the entire surface and this was followed by incubation at 37 C. for 20-24 hours. The resulting growth was then suspended by careful shaking in salt solution to the amount of 10 c.c. Injections were made of 0.5 c.c of this suspension, equal to 1/20 slant.

Rabbits were selected weighing from 2,800 to 3,500 gm., and apparently healthy. Preliminary fecal examinations to insure absence of protozoa or other parasites were not made.

Direct injection into the gallbladder was made as follows: The animal, stretched on a Latapie operating board, was prepared by shaving the abdomen, after which the surface was painted with tincture of iodine. A few moments later this was washed off with cresol solution. The fur coat about the shaved area was also wet down well with cresol, and the animal was covered entirely with a sterile sheet having a circular window over the site of incision.

The incision, generally 1 inch long, is made just at one side of the median line with its anterior end at the base of the sternum. Ordinarily by use of a retractor and sponges, the gallbladder located in its cleft is revealed without difficulty. In case it is distended it should be evacuated partially by the application of gentle pressure from the finger. By smooth, blunt forceps, the bladder may be drawn forward gently and injection carried out by an assistant. The needle ordinarily used is No. 27 gage, 1/2 inch long. The fine needle must be inserted through the outer aspect of the gallbladder wall where the blood vessels are not so numerous. On withdrawal of the needle it is wise to watch for a moment in order to make certain that there is no leakage or bleeding after the abduction has ceased. The incision through the abdominal wall is then sutured, skin clips are applied, together with dressing of zinc oxide ointment, sterile cotton and collodion. Using these precautions, together with daily inspection, the loss of animals will be negligible.

By this technic, 16 animals were inoculated directly into the gallbladder, while as a check 9 received the same dosage in the posterior ear vein. Of the 16 first mentioned, 8 were bled regularly for agglutinin titration, while the other 8 were not tested so systematically.

⁶ Arch. Int. Med., 1913, 12, p. 613.

Tables 1 and 2 give the agglutinin titers of rabbits with intracystic injection as compared with those treated intravenously. Titers were read after 2 hours' incubation at 37 C. followed by a night, about 18 hours, in the icebox. Only such tubes as showed complete clarity were considered. The antigen was living bacilli from a 24-hour slant suspended in sterile salt solution.

TABLE 1
AGGLUTININ TITERS OF RABBITS INJECTED DIRECTLY INTO THE GALLBLADDER WITH 1/20
SLANT CULTURE OF *B. TYPHOSUS*

Rabbits	3 Days	5 Days	7 Days	11 Days	14 Days	21 Days	28 Days	35 Days
804	10	40	80	80	20	80	40	10
633	>5	40	40	40	40	40	40	20
684	>5	160	640	160	160	160	80	80
614	5	160	80	80	80	40	40	40
661	5	160	160	160	160	80	80	40
620	5	80	160	80	40	40	20	20
680	>5	80	80	80	80	40	20	20
644	10	10	20	160	80	80	20	10
427	320	...	160
895	160	80
679	..	40	...	20
870	160	...	80
829	10	...	20
898	..	80	40
876	40	20
817	10	40	160	1280	Dead

> signifies "less than."

TABLE 2
AGGLUTININ TITERS OF RABBITS INJECTED INTRAVENOUSLY WITH 1/20 SLANT CULTURE
OF *B. TYPHOSUS*

Rabbits	3 Days	5 Days	7 Days	11 Days	14 Days	21 Days	28 Days	35 Days
826	20	...	320	80	320	80	40	40
652	10	...	80	80	80	40	40	20
674	10	...	2560	320	640	320	160	80
802	20	...	320	320	320	80	40	20
698	10	...	80	40	80	80	80	40
365	5	320	320	160	160	160	Dead	...
175	0	20	80	40	20	80	Dead	...
366	0	320	320	640	80	160	Dead	...
501	..	5	20	40	40	Dead

From these experiments, it is evident that the placing of *B. typhosus* within the gallbladder is followed by prompt mobilization of antibodies. The resulting titer is not so high as that following intravenous injection of a comparable amount, but nevertheless it is unmistakable.

Following this series it was determined to ascertain whether injection of dead typhoid bacilli into the gallbladder is followed by agglutinin production. The same technic as that described was used. The sus-

pension had been heated to 56 C. for 45 minutes and then tested for sterility by cultural methods. The volume injected was the same as before. The results are shown in table 3.

It is evident that implantation of dead typhoid bacilli within the gallbladder stimulates the appearance of agglutinins in the circulation, promptly and vigorously.

The results seem to indicate that the bacilli must pass through the cystic wall since it is unlikely that the mere presence of *B. typhosus* within the cystic bile itself can stimulate such heavy agglutinin production in the circulation. Moreover, it is stated by Gay⁷ that only approximately two-thirds of chronic human gallbladder carriers of typhoid give an agglutinin reaction, which of itself probably indicates that the assumption noted is correct. It is a well-known anatomic condition that although the blood circulation in the gallbladder wall is not copious compared to certain other tissues, the lymphatic channels

TABLE 3
AGGLUTININ TITERS OF RABBITS INJECTED DIRECTLY INTO THE GALLBLADDER WITH 1/20
SLANT CULTURE OF *B. TYPHOSUS* KILLED BY HEAT

Rabbits	3 Days	5 Days	7 Days	11 Days	14 Days	21 Days	28 Days	35 Days
628	..	40	160	20	40	40	80	40
612	5	40	160	40	40	20	40	40
667	10	1280	640	160	160	160	80	160

are numerous. Thus it is probable that if there be dissemination of the bacterial forms after passing through the bladder wall, the lymph is instrumental in part at least.

Since it seems likely that particulate matter such as typhoid bacilli may pass through the wall of the gallbladder, it was decided to study whether this is possible. The method was first to inject a large quantity of typhoid bacilli directly into the cystic bile through the bladder wall and then by bleeding at intervals to search for the bacilli in the circulation. Following this, after a few hours the animal was killed and a bacteriological examination of various tissues made. This examination was carried out as follows: After chloroforming, the body was opened; portions of the organs were removed with sterile instruments, macerated in sterile salt solution with mortar and pestle, after which approximately 1 c.c. of the resulting suspension was placed in plain broth tubes. Ordinary routine of cultivation on solid mediums followed by

⁷ Typhoid Fever Considered as a Problem of Scientific Medicine, 1918, p. 129.

agglutination tests with known antityphoid serum was used for recognition of bacterial growths. Descriptions of these experiments follow:

Exper. 1.—Male rabbit 300, 3,500 gm., injected by laparotomy with 0.5 cc suspension containing 1/6 slant of a 24-hour growth of *B. typhosus* on rabbit blood agar. The animal was bled 1/2 hour after injection and 0.5 cc of the fresh blood placed in plain broth. Bleedings were made hourly thereafter until death by chloroforming at 5 1/2 hours. At necropsy cultural examinations were made from heart blood, bile, urine, duodenal content, liver, kidney, spleen, lung and bone marrow. *B. typhosus* was recovered from liver, spleen, lung, bile and intestinal content but not in any other preparations.

Exper. 2.—Male rabbit 350, 2,800 gm., underwent laparotomy with direct injection of 0.5 cc containing 1/4 of a 24-hour slant culture of *B. typhosus*. One-half cc of blood was removed from the ear vein 1/4 hour after injection and placed in plain broth. Like bleedings were made hourly thereafter until time of necropsy following chloroform at 5 1/4 hours. Cultural preparations were made from heart blood, bile, urine, duodenal contents, liver, spleen, kidney, lung and bone marrow. *B. typhosus* was obtained from bile and duodenal contents, liver, spleen, kidney and lung. All other preparations were negative.

Exper. 3.—Male rabbit 374, 3,500 gm., received 0.5 cc suspension of 1/4 of a 24-hour rabbit blood slant culture of *B. typhosus* directly into the gallbladder. Following this 0.5 cc blood was removed from the ear vein 1/4 hour later and thereafter hourly to the time of necropsy following death by chloroform, at 6 1/4 hours. These samples were placed immediately in plain broth. At final dissection cultural examinations were made of heart blood, bile, urine, duodenal content, liver, spleen, kidney, bone marrow and lung. *B. typhosus* was recovered from the bile, duodenal content and spleen. All other cultures were negative.

It is evident that *B. typhosus* after intracystic injection may pass promptly through the epithelial lining of the bladder and be disseminated to various organs of the body. The lymph would seem to be the medium for carriage, as the bacillus was not isolated from the peripheral blood stream during any of the 3 experiments. It should be noted, however, that during the earlier stages of this work and while proper technic was being elaborated, 3 small rabbits of 1,500 to 1,800 gm. weight were injected directly into the gallbladder. These died between the tenth and eighteenth hour after operation. Rigor mortis had set in by the time of necropsy, but no more exact statement can be made regarding the period between death and examination. *B. typhosus* was isolated from the heart blood of each one. In these dead animals, therefore, the organism was present within the circulation but we have no assurance that its presence was not due to postmortem migration.

Microscopic examination by paraffin section stained by hematoxylin—eosin after Zenker or Kaiserling was made of the gallbladder of animals having undergone laparotomy and intracystic injection. The

results are similar to those of Findlay and Buchanan,⁸ Bindseil,⁹ Goebel¹⁰ and Messerschmidt,¹¹ with human material and those of Meyer, Neilson and Feusier¹² with animals. Two weeks had elapsed between the injection and the examination. Sections showed necrosis accompanied by desquamation of the epithelium, especially on the outer folds of the villi, although it was present to less marked degree at their base. This change was evident both in the body and in the neck of the bladder. Areas of intense infiltration were present in the villi and to a less degree in inner portions of the tunica propria. Numerous capillaries were blocked by masses of erythrocytes. Edema was marked and the wall thickened. Although Meyer, Neilson and Feusier¹² state that rabbits injected with *B. typhosus* into the gallbladder showed no stripping of epithelium, it will be noted that those of the series described here did. It is very possible that the time factor explains this difference. The picture was the same whether with intravenous or intracystic injection.

SUMMARY

Injection of *B. typhosus* directly into the gallbladder of the rabbit is followed by prompt appearance of agglutinins within the circulation. This response is stimulated by dead as well as by living organisms. Typhoid bacilli implanted within the gallbladder pass through the lining epithelium and appear shortly in other organs, such as the spleen, liver, lung, and kidney, but they were not isolated from the peripheral circulation. It seems likely that lymph is at least partially responsible for this distribution. Migration of *B. typhosus* through the epithelium into the tunica is attended after appropriate lapse of time by the formations of lesions which are characteristic of those found in the typhoid carrier.

⁸ Glasgow Med. Jour., 1906, 65, p. 177.

⁹ Ztschr. f. Hyg. u. Infektionskr., 1913, 74, p. 369.

¹⁰ Ibid., 1914, 78, p. 555.

¹¹ Ibid., 1913, 75, p. 411.

¹² Jour. Infect. Dis., 1921, 28, p. 76.

RESPIRATORY INFECTION AND SEPTICEMIA OF CATS DUE TO THE HEMOLYTIC STREPTOCOCCUS

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In February, 1921, an acute respiratory disease suddenly broke out among 25 cats kept in a room in this laboratory. The disease spread so rapidly and was so severe that all of these animals except 2 were dead within 15 days. This epizootic, having features in common with epidemics of respiratory disease in man, was an example of the spontaneous contagious infections occurring among laboratory animals which are becoming of increasing use to the experimental epidemiologist.¹

The organism which caused the disease was a hemolytic streptococcus. Cats are said to be relatively insusceptible to infection with hemolytic streptococci, and a record of only one similar epizootic has been found in the literature. This occurred in a laboratory in Leyden, and was reported by de Jong,² in 1912. The "streptococcosis" of cats which he described appears to have been identical with the disease which occurred here. From his description, however, it is not possible to recognize the type of streptococcus which infected his animals.

The 25 cats among which the disease broke out here were kept as "stock" in a warm room. They were not in cages. As new cats were placed in this room at irregular intervals, it is not possible to say when the animal or animals introducing the infection were mixed with the others. When attention was directed to the condition, several cats were found to be sick, and in a few days all of the animals except 2 had become infected. The onset of the disease was characterized by sneezing, the appearance of a thin greenish discharge from the nose and by conjunctivitis. The infected animals soon became prostrated, lost weight rapidly, became exhausted and stuporous and died after 4 or 5 days. The cats had a leukocytosis of 50,000 to 100,000 cells per cubic millimeter. The percentage of polymorphonuclear neutrophils varied from 73 to 90.

Received for publication, Aug. 3, 1922.

¹ Flexner, S.: *Experimental Epidemiology*, Jour. Exper. Med., 1922, 36, p. 9.

² *Centralbl. f. Bakteriol.*, I, O., 1912, 66, p. 281.

At necropsy all of the cats presented the same general appearance. They were emaciated. A green mucopurulent exudate filled the cavities of the nose and extended over the nasopharynx down into the trachea as far as its bifurcation. The cranial sinuses were normal, and there were no gross or microscopic lesions attributable to this infection in any other organs. No evidence of pneumonia was found when the lungs were examined.

In smears of the exudate from the nose, pharynx and trachea, there were numerous gram-positive cocci in chains. This organism always predominated, and it was usually the only organism seen in the smears of the exudate from the trachea. Cultures of the exudate yielded many colonies of *Streptococcus haemolyticus*. Bacteriologic examination of the blood of the cats before and after their death revealed a more adequate basis for the severity of their symptoms. After the first day of the disease, all animals had a septicemia due to *Streptococcus haemolyticus*. Streptococci were sufficiently numerous in the blood to be recovered by plating a single drop of blood. Pure culture of the hemolytic streptococcus were obtained from the heart blood of these cats.

The disease, then, was characterized by an infection of the upper respiratory tract, followed shortly by a fatal septicemia. The absence of pneumonia was striking. Search for organisms of the hemorrhagic septicemia group was made. No evidence was obtained that such organisms were concerned in this infection. *Streptococcus haemolyticus* was predominant in the lesions and was not associated with any other bacterium in causing the septicemia.

Strains of this streptococcus were isolated from the trachea of 2 cats and from the blood of 7 cats. They were studied culturally and immunologically.³

STREPTOCOCCUS HAEMOLYTICUS FROM CATS

Morphology and Staining.—The streptococcus formed long chains of 8 or 16 or more cocci in meat infusion broth. The size of the cocci varied considerably, though not unusually. In some chains, the apposed sides of the cocci were flattened. All strains were gram-positive.

Cultural Studies.—The streptococcus grew well aerobically on blood agar, serum mediums and in meat infusion broth. On an agar plate containing 5% rabbit blood, the streptococcus formed a wide zone of

³ These strains were isolated by the late Dr. H. B. Cross. In the subsequent studies, the work of Miss Tuthill was of great assistance.

clear hemolysis—the characteristic beta-type of Brown. When 1 c c of a 16 to 18-hour broth culture was added to 1 c c of 5% sheep cells, and incubated at 37 C., the cells were completely laked in a short time.

The fermentation reactions were determined by using mediums containing serum-water and 0.5% carbohydrate, with brom-cresol-purple as the indicator. These mediums were sterilized in the Arnold sterilizer on 3 days. The cultures were kept in the incubator at 37 C. for 2 weeks under daily observation. All strains fermented the same “sugars”—forming acid from dextrose, levulose, galactose, maltose, lactose, sucrose, salicin, raffinose, arabinose and dextrin. None fermented mannitol or inulin. These fermentation reactions are characteristic of Holman's *Streptococcus pyogenes*.

The final P_H produced by these streptococci was determined by growing them in 1% dextrose meat infusion broth. Each tube contained 10 c c of the medium, the initial reaction of which was P_H 7.8. These cultures were incubated at 37 C. for 24, 48 and 72 hours. Abundant growth occurred. After 48 hours the maximum acidity had been produced, and the reaction, determined colorimetrically with methyl red, was P_H 4.8. This reaction approaches that produced by the hemolytic streptococci from cattle.

Hydrolysis of Sodium Hippurate.—Recently, Ayers and Rupp⁴ have shown that beta-hemolytic streptococci from bovine sources decompose sodium hippurate to benzoic acid and glycocholl while the beta-hemolytic streptococci of human origin are unable to effect this hydrolysis. The medium was prepared by adding 1% sodium hippurate to meat infusion broth containing a trace of ferric chloride. This was sterilized in the autoclave. All the strains of streptococci from cats and, as controls, a human strain and a characteristic bovine hemolytic streptococcus (No. M43 from Dr. F. S. Jones of the Department of Animal Pathology of the Rockefeller Institute) were inoculated into tubes of this medium. Tests for benzoic acid were made after the cultures had been growing for 24 hours. The reagents used for these tests were: (1) 12% ferric chloride solution in 2% hydrochloric acid and (2) 50% sulphuric acid. To 2 c c lots of the clear culture fluid, 0.5 c c of each reagent was added. The results were as follows:

1. Hemolytic streptococci from cats: No hydrolysis of sodium hippurate.

⁴ Jour. Infect. Dis., 1922, 30, p. 80.

2. Hemolytic streptococcus, human type: No hydrolysis of sodium hippurate.

3. Hemolytic streptococcus, bovine type, M_{43} : Hydrolysis, shown by persistent brown precipitate with ferric chloride and precipitation of crystals of benzoic acid with H_2SO_4 .

Summary of Cultural Reactions.—All the strains of hemolytic streptococci isolated from the exudates and blood of these cats had identical cultural reactions. They caused the characteristic beta-type of clear hemolysis on blood agar, failed to ferment mannitol and inulin, produced a final P_H of 4.8, and were unable to hydrolyze sodium hippurate. These reactions seem to exclude them from the group of bovine hemolytic streptococci but do not indicate what position they occupy in the scheme of classification.

IMMUNOLOGIC REACTIONS

Agglutination and the absorption of agglutinins were used in the attempt to discover the immunologic relationships of these streptococci from cats. As usual, it was difficult to make homogeneous suspensions of these organisms. Satisfactory suspensions were sometimes obtained with cultures in an infusion broth prepared from beef-heart. But the method of using cultures from ascites fluid agar, described by Herrold⁵ was found to be much more satisfactory. Only homogeneous suspensions were used. As controls in the agglutination tests, a suspension of each organism in broth or salt solution was incubated with the other tubes in a water bath at 56 C. for 2 hours. Concentrated emulsions of streptococci grown on ascites fluid agar were used for the absorption tests. The following agglutinating serums were used:

1. Monovalent agglutinating serums for the human types of hemolytic streptococci, S 3, S 23 and S 84.⁶ These were obtained from Dr. Dochez.

2. Monovalent agglutinating serum specific for a strain of hemolytic streptococcus from scarlet fever, prepared by Dr. Bliss.

3. Polyvalent antistreptococcus serum, from a horse, prepared by Dr. Huntoon.

4. Monovalent agglutinating serum for the bovine type (M_{43}) of hemolytic streptococcus, prepared by immunizing a rabbit to this strain. Titer 1:1,600.

⁵ Jour. Infect. Dis., 1922, 30, p. 80.

⁶ Dochez, A. R.; Avery, O. T., and Lancefield, R. C.: Jour. Exper. Med., 1919, 30, p. 179.

5. Agglutinating serums specific for the hemolytic streptococci from cats, prepared by immunizing rabbits with many injections during about 6 weeks, as follows:

No. 153—antistreptococcus from blood of cat A; titer 1:5,120.

No. 154—antistreptococcus from trachea of cat A; titer 1:2,560.

No. 155—antistreptococcus from blood of cat B; titer 1:2,560.

No. 156—antistreptococcus from trachea of cat B; titer 1:1,280.

No. 157—antistreptococcus from blood of cat G; titer 1:2,560.

The results of the agglutination tests, with omission of the long protocols, are summarized in table 1.

These results show that the group of streptococci from these cats is composed of identical strains, and that they are immunologically

TABLE 1
AGGLUTINATION REACTIONS OF HEMOLYTIC STREPTOCOCCI FROM CATS

Serum	Result
Antistreptococcus serum:	
Human Type S 3.....	No agglutination of streptococci from cats.
Human Type S 23.....	No agglutination of streptococci from cats.
Human Type S 84.....	No agglutination of streptococci from cats.
Human scarlatinal Type.....	No agglutination of streptococci from cats.
Polyvalent serum (Huntoon).....	No agglutination of streptococci from cats.
Bovine type (M_{43}).....	No agglutination of streptococci from cats.
No. 153, cat A, blood.....	Agglutinated all strains from cats.
No. 154, cat A, trachea.....	Agglutinated all strains from cats.
No. 155, cat B, blood.....	Agglutinated all strains from cats.
No. 156, cat B, trachea.....	Agglutinated all strains from cats.
No. 157, cat G, blood.....	Agglutinated all strains from cats.

different from human and bovine types of hemolytic streptococci. They form a cultural and serologic unit, exhibiting the homogeneity of an "epidemic strain," which is entirely concordant with their unvarying effect on the animals from which they were isolated.

The absorption of agglutinins confirmed the results of the agglutination reactions. To absorb the agglutinins, a thick emulsion of streptococci was made by washing off the cultures from slants and plates of ascites fluid agar with salt solution and by centrifuging this. The antisera were diluted 1:20, and 2 volumes of serum were mixed with 1 volume of sedimented streptococci. This mixture was incubated in a water bath at 56 C. for 2 hours. The serum was then freed from the organisms by centrifugation, and used for agglutination tests. The results of these are summarized in table 2.

Pathogenicity.—The streptococci soon lost the ability to produce the disease when placed within the noses of cats. Their pathogenicity for

cats, however, was retained through a number of transfers in broth. Three cc of an 18-hour broth culture, injected intravenously, produced septicemia which killed the animals in 72 hours. The opportunity has not presented itself for an extended "epidemiologic" study with this streptococcus.

Incidence of Streptococcus in Cats.—To determine the frequency of the occurrence of streptococci in the throats of cats, 50 normal animals were studied. Some of the animals were taken from the stockroom, others used as soon as they were brought into the laboratory from the streets. The throats were swabbed and the material thus obtained was plated on 5% rabbit's blood agar. A beta-hemolytic streptococcus was found in only 1 of these cultures, showing again that streptococci occur only rarely in the nasopharynx of cats (0.5%).

TABLE 2
RESULT OF AGGLUTINATION TESTS

Antistreptococcus Serum	Absorbed with	Agglutination Test after Absorption	Result
153 antistreptococcus Cat A, blood	Streptococcus from cat A blood	All strains of streptococci from cats	Removed agglutinins for all strains of streptococci from cats
153 antistreptococcus Cat A, blood	Human Type streptococcus	All strains of streptococci from cats	Slight, insignificant reduction of agglutins
153 antistreptococcus Cat A, blood	Bovine type streptococcus M ₄₃	All strains of streptococci from cats	Slight, insignificant reduction of agglutins

SUMMARY

An unusual epizootic in cats due to a variety of hemolytic streptococcus is described. The disease began as an infection of the upper respiratory tract and ended with septicemia. Pneumonia did not occur. The infection spread rapidly among associated animals and had a high mortality.

The organism causing the disease, *Streptococcus haemolyticus*, resembled the human types of hemolytic streptococci more closely than the bovine, but different immunologically from the strains of both of these varieties, with which they were compared. All the strains of this streptococcus were identical.

MODIFIED GRAM STAINS

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The Gram stain continues to be an ever fruitful subject for experimentation. The literature reveals a variety of methods, many of which have proved valuable. The Society of American Bacteriologists, through the reports of its committee, has offered 2 methods for consideration which have been widely used.¹ More recently, however, 2 important modifications of the Gram stain have been published. The first by Atkins² proposes the use of aniline sulphate in place of aniline, and a stronger iodine solution, to which sodium hydroxide has been added. The other method is that described by Burke.³ Here the chief points of interest are the use of: a 1% aqueous solution of dye, sodium bicarbonate, and acetone as a decolorizer. Both Atkins' and Burke's methods have yielded satisfactory results in our hands, and it would be difficult to enter into a discussion of the relative merits of these methods without previous exhaustive investigation. Suffice it to say that we have taken the liberty of combining some of the principal features of each of these methods in an attempt to economize time and expense without sacrificing the quality of the results. Before advancing the reasons for choice we present the method in detail step by step as used by us.

1. Air dry a thinly spread film and fix with least amount of heat necessary to kill the organisms and fix them to the slide.

2. Flood slide with dye solution. This is prepared by previously mixing in a beaker about 30 drops of a 1% aqueous solution of methyl violet 6 B (Coleman and Bell) with 8 drops of a 5% solution of sodium bicarbonate. Allow the mixture to remain on slide 5 minutes or more.

3. Flush off the excess stain with the iodine solution and cover with fresh iodine solution for 2 minutes or longer. The iodine solution consists of 2 gm. iodine dissolved in 10 cc normal sodium hydroxide solution, to which is then added 90 cc of water.

Received for publication Aug. 3, 1922.

¹ Jour. Bacteriol., 1919, 4, p. 107.

² Ibid., 1920, 5, p. 321.

³ Ibid., 1922, 7, p. 159.

4. Drain off the excess iodine solution, without blotting (no water being used), but the film is not permitted to become dry.

5. Add acetone (100%) drop by drop until no color is seen in the drippings from the slide, which is slightly tilted. This usually requires less than 10 seconds and should be reduced to a minimum.

6. Air dry the slide.

7. Counterstain for 10-30 seconds with 0.1% aqueous solution of basic fuchsin.

8. Wash off excess stain by short exposure to tap water and air dry. If slide is not clear immersion in xylol has been recommended.

The advantages from such a procedure are considered separately in the papers of Atkins and Burke and may be briefly enumerated as follows:

In step 2 a 1% aqueous solution of violet (either methyl 6 B, crystal or gentian violet) is easily prepared and has relatively good keeping qualities. Sodium bicarbonate neutralizes acidity and improves the intensity of the stain in the gram-positive organisms. We have found that the more intimate mixture of sodium bicarbonate and dye solution prior to application yields somewhat better results than when these are used separately. A stock solution of dye and bicarbonate cannot be made up since deterioration sets in rapidly. Fortunately there is no danger of overstaining provided that actual drying does not occur. This does not apply to the counterstain.

In step 3 of this iodine formula, sodium hydroxide is used to dissolve the iodine. This is not only economical, but also yields free hydroxyl-ions which may aid in intensifying the stain.

In steps 4, 5 and 8 it will be noted that we do not use blotting paper at any stage in the procedure because it not only appears to be superfluous but may frequently leave fibers on the slide, which are undesirable.

In step 5 acetone has proved to be more satisfactory than either 95% or absolute alcohol and is also more economical as regards expense and quantity.

In step 7 we prefer fuchsin as a counterstain because it is bright and gives an excellent contrast.

The Gram stain described has been used with particularly gratifying results in connection with our studies on the therapeutic effect of *Lactobacillus acidophilus*.⁴ The daily routine required the staining of Lacto-

⁴ Kopeloff, Nicholas and Cheney, C. O.: Proc. Soc. Exper. Biol. Med., 1922, 19, pp. 372-373. Jour. Am. Med. Assn., 1922, 79, p. 609.

bacillus acidophilus in milk. Using the Sterling modification of the Gram stain with alcohol as a decolorizing agent, the *débris* was dark purple which made it somewhat difficult to identify individual bacilli. However, with the procedure here outlined the *débris* is pale pink, which causes the gram-positive bacilli to stand out sharply. Furthermore, the gram-negative *débris* does not obscure the presence of gram-negative organisms as gram-positive *débris* is likely to do. Again the routine examination of feces in these studies⁴ was attended with the usual difficulties when the Sterling modification of the Gram stain was employed. The microscopic examinations therefore were greatly facilitated by the present procedure, which not only rendered the *débris* gram-negative but yielded a sharper differentiation between gram-positive and gram-negative organisms.

In order to widen the scope of its application a number of common pathogenic and saprophytic bacteria were stained by this method. It was found that the organisms were stained in a way that left little to be desired with regard to the Gram phenomenon or morphology. Particular attention may be directed to the clear cut results obtained with gonococcus and the diphtheric bacillus which ordinarily present considerable difficulty in Gram staining.

SUMMARY

A method of Gram staining has been outlined which is based on the results obtained by the use of modifications devised by Burke³ and Atkins.²

The method has yielded particularly good results in staining milk slides for *Lactobacillus acidophilus* and in staining fecal specimens. By this method, the gonococcus and the diphtheric bacillus are well differentiated and more easily identified than by the older methods. The same was found to be true for a number of common pathogenic and saprophytic bacteria.

VARIATION AND LIFE CYCLES OF PATHOGENIC BACTERIA

ONE PLATE

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In his "Species Plantarum," Linné points out the great variability of fungi. He found it difficult to recognize and limit the species. For the pathogenic bacteria this difficulty continued a much longer time. Nägeli denied the existence of constant species and believed that the same species produced lactic acid and caused scarlet and typhoid fevers and other diseases. In his laboratory the cultures of *B. subtilis* were changed to *B. anthracis*. R. Koch¹ deserves great credit for proving the constancy of many pathogenic bacteria. In the earliest studies he discovered the endogenous spores of *B. anthracis* and mentioned several extraordinary forms. He evidently soon lost interest in such forms, however, and now their existence is denied by most bacteriologists or they are considered degenerative products or involution forms. Thus science has gone from one extreme to the other; scientists have striven to make Nature less complicated than it is.

Löhnis,² in an important work in 1921, cites a great number of authors, some earlier than Koch, who have studied the atypical forms of bacteria. Many are easily recognized from plates and descriptions. The descriptions of variation and life cycle of a species are, however, difficult to interpret. For such observations the pure culture is necessary.

In 1881, I studied *B. typhosus* in patients and found in the living blood both motile and immotile rods and in the dead human body the oval rods of Eberth-Koch. The following year, I had to combat a great epidemic of typhoid fever. The observations made gave me ideas for a study of *B. typhosus*, which I still continue. I then came to the conclusion that neither the pathogenicity of this organism nor the epidemiology of typhoid fever can be fully explained until the life cycle of *B. typhosus* has been thoroughly investigated.

Received for publication, Aug. 8, 1922.

¹ Zur Untersuchung von pathogenen Organismen, Mittl. d. Keis. Gesundh. Amts., 1881, 1, p. 1.

² Studies upon the Life Cycles of the Bacteria, Memoirs National Acad. Sciences, 1921, 16, p. 335.

LIFE CYCLES OF SOME PATHOGENIC BACTERIA

In the first two decades of my studies I occasionally found new forms of great interest, for example, plasmodia and microconidia of *B. typhosus*; but I was not able to produce them ad libitum. Now I conclude that low temperature had effected these results. Since 1908 I have used, to a great extent, low temperature and drying mediums for my cultures. Thus it is easy to produce the new varieties in great abundance. Burri's method of one-cell culture has further advanced my studies. In my laboratory Troili-Peterson³ successfully modified Burri's method.

It is quite necessary to discern common variation from the constant variety that is never able to go back to the original. Linné speaks both of variation under the influence of environment and of constant varieties. He found the latter numerous among the higher plants, and he was not able to reproduce the original form by culture. Linné's principle is obvious, but in order to prove the constancy of a bacterial form, one must work for years. Nobody can pretend to know the complete life cycle and all the varieties of a bacterial species. It would be an assumption to think so, at least in our day.

The important varieties must be sharply distinguished from all degenerated or dying forms. Thus each variety must be examined to determine its origin and its development. The literature of bacteriology is abundant in descriptions of extraordinary bacterial forms without concise statements; frequently it was not even determined whether the forms were living or dead.

B. typhosus.—On drying substrate at 10-14 C. the common rods become transformed in a remarkable manner; they grow larger and often produce exogenous globules terminally and laterally; the membranes seem to dissolve; the protoplasm protrudes; the rods lose their form and produce protoplasmic masses, plasmodia. The exogenous conidia germinate immediately to new rods and globules; sometimes I saw them motile (figs. b, d and e).

We often find small immotile free granules in great numbers. They are probably produced when the substrate is poor, dry and cold. Figure c presents beautiful lateral microconidia, a rare occurrence.

All is rapidly changed when the transformed culture is transferred to a good substrate at 37 C. The rods sprout and produce large conidia that germinate immediately. The large globules, acting as sporangia,

³ Ein-Zell-Kultur von langsam wachsenden Bakterienarten, etc., Centralbl. f. Bakteriöl., ii, 1914, 42, p. 526.

open; globules and fine curved chains appear. The contents look as shown in f; the cell wall is often visible at the side. The whole phenomenon occurs within a few hours. The next day we see only common rods.⁴

At low temperature, e. g., in broth, the myceloid prevails. We find long, thick, intricate threads that often produce large conidia. The long, thin threads formed in poor nutritive solution are inclined to bud to microconidia.

In all cultures we find needle-shaped rods, fine and motile, 2 microns long. On poor substrates the rods are often very frequent and keep their constancy fairly well. I think they are of the same origin as the microconidia.

Eberth and Koch saw in the dead human body chiefly aggregates of large oval rods, 2 microns long, which took the stain weakly. This variety is remarkable, though almost forgotten. Perhaps it constitutes a middle form between plasmodium and common rod.⁵

All examined varieties of *B. dysenteriae* agree with those of *B. typhosus*. The life cycles are similar but not identical. *Spirillum cholerae* agrees also strikingly with *B. typhosus*. At 14-15 C. on drying substrates the spirilla develop quickly into thick and long forms and reproduce through budding conidia which promptly germinate. At the same time small amorphous plasmodia appear (figs. i and 1). After a month the threads look empty, and the culture is replete with germinating conidia. The conidia are often motile; however, they are immotile at low temperature and are then more easily observed. It is interesting to follow the growth of the conidia to spirilla; usually one spirillum breaks forth, sometimes two from the same globule. The next day only small spirilla are to be found in the culture.⁶

In the year 1852, Perty⁶ discovered both endogenous spores of a bacillus and exogenous conidia or sporangia of *Spirillum undula*. He saw the spirilla move with lateral and terminal globes, having a diameter of 2 microns and quite distinct outlines. Nevertheless, Perty declared that the exogenous bodies were coagulated clumps.⁷ Beyond doubt they were able to sprout.

⁴ Almquist, E.: Studien über das Verhalten einiger pathogenen Mikroorganismen, Centralbl. f. Bakteriöl., I. O., 1908, 48, p. 175. Wuchsformen, Fruktifikation und Variation der Typhusbakterie, Ztschr. f. Hyg. u. Infektionskr., 1916, 83, p. 1.

⁵ Footnote 1, p. 45. Gaffky: Zur Aetiologie des Abdominaltyphus, Mitteilungen d. Kais. Gesundh. Amts., 1884, 2, p. 372.

⁶ Zur Kenntnis kleinster Lebensformen, 1852.

⁷ Ibid., p. 132.

In laboratory cultures of *Sp. cholera* we find many large globules after a short time. So far as I know, no one has studied their origin or development. Kitasato⁸ apparently believes that they are dead. I believe so also. Ferran and Hüppe describe forms of *Sp. cholera* similar to arthrospores but without resemblance to the exogenous germinating conidia. Gamaleia⁹ used substrates containing lithium and saw many globules and other forms replete with crystals of lithium. He found it difficult to ascertain whether these globules were living.¹⁰

Olsson¹¹ made interesting studies of the life cycle of the same spirillum. In certain mediums they were long and immotile; the colonies were not smooth but wrinkled. Gradually all the organisms assume this changed form permanently. The transformation is most rapid at about 20 C.; it can be completed within half a year. It is surprising that these immotile spirilla return to the normal form when grown for one month on agar at 37 or at 17 C. Olsson found that the virulence was then increased.¹¹

B. diphtheriae.—I have used long, granular, virulent rods from single cell cultures (fig. N). On a lactate substrate the rods transform themselves within 2 or 3 weeks at 19 or 26 C. into fine chains, similar to oidia (fig. o, p). They live on the same substrate several months as a kind of resting form and are easily observed after having been stained. When the oidia are transferred to serum at 37 C. they grow immediately to typical rods. After one day all are changed to rods.

In drying broth the rods are transformed into large masses within a month at 35 and at 17 C. In addition to clumps we see also thick rods with branches and exogenous buds. Such cultures rapidly produce fine forms on serum at 37 C. (fig. r). Within a few days only normal rods are visible (N).

The virulence increases by passing through the described cycle. Koraen and I¹² were also able to make a culture of very fine needles 1-2 microns in length which was quite virulent. In drying broth the latter strain turned partially into large masses within 7 weeks at 22 and also at 18 C.⁶

Many authors have described other forms of *B. diphtheriae*. Only by studying single-cell cultures, will their importance be revealed.

⁸ Die Widerstandsfähigkeit der Cholera Bakterien gegen Eintrocknen, Ztschr. f. Hyg. u. Infektionskr., 1889, 5, p. 34, and 6, p. 11.

⁹ Elemente der allgemeinen Bakteriologie, 1900.

¹⁰ Ibid., p. 215.

¹¹ Zur Variation des Choleravirus, Centralbl. f. Bakteriöl., I., O., 1915, 76, p. 23.

¹² Studien über Biologie und Wuchsformen der Diphtheriebakterie, Ztschr. f. Hyg. u. Infektionskr., 1918, 85, p. 347.

Micrococcus thulini.—This coccus appeared in Stockholm during the Spanish fever in 1917 and grew in chronic wounds and in the throat as a streptococcus. On agar the same form turned into a large staphylococcus-like form (fig. S). After a short time the cultures began again to reproduce small streptococci and soon lost their vitality (fig. t). In broth at 35 C., the small cocci are always more numerous (fig. u).

On drying agar we find very small buds on the micrococci; *M. pyogenes-aureus* will act in the same way under such circumstances. The buds constitute a kind of microconidium and develop rapidly into cocci on good substrates. After passing the cycle, the small cocci reproduce the large cocci (fig. S). From Koraen's¹³ observations we conclude that *M. thulini* gains new energy in passing the cycle.

BACTERIAL MUTATION

The study of this important question began when Jenner discovered cowpox. Today, after a great deal of experience, we know that the virus of cowpox does not cause smallpox. This virus has been transformed in the body of the cow and causes another disease with shorter incubation. Thus a constant variety, a real mutation, has appeared.

B. typhosus.—In the year 1910 I began to separate with the Berkefeld filter the small granules that I often encountered in my saprophytic cultures. From 100 experiments I 10 times obtained cultures of filtrable bacteria that did not go back to the origin, *B. typhosus*. These strains produce in the rabbit a serum that agglutinates *B. typhosus* in a dilution of 1:500 and also gives good effect in Pfeiffer's test. I have called them *B. antityphosum*.¹⁴

I have cultivated these strains for 11 years and have never seen them revert; they are beyond doubt constant. At low temperature and on poor substrate they grow more readily than *B. typhosus*; therefore I thought it might be possible to separate the granules from the rods in pure cultures of *B. typhosus*. Sometimes I succeeded in isolating the granules by culture.

A third method has also occasionally given positive results, i. e., new vigorous constant forms were obtained. After heating at 60 C. the appearance is much changed, the plasmodia prevail, and the rods assume monstrous shapes. They agglutinate somewhat like *B. typhosus*.

¹³ Studien über Umformung von Mikrokokken in trocknender Kultur, Ztschr. f. Hyg. u. Infektionskr., 1918, 85, p. 359.

¹⁴ Studien über filtrierbare Formen in Typhuskulturen, Centralbl. f. Bakteriol., I, O., 1911, 60, p. 167.

Different strains are gained by filtering, culturing and heating; but the same method gives different forms. I obtained different granules by filtering and culturing. The granules are different also in the cultures of *B. typhosus*. In the plate the single microconidia are not similar to the curved chains in figures f and g. In cultures I obtained strains more similar to the free granules. On the other hand, *B. antityphosum* appears both as curved chains and as free granules, sometimes forming a short chain. To be sure, *B. typhosus* produces by mutation several different varieties.

Spirillum cholera.—By heating at 60 C. I have received some new varieties. One of them I have studied for about 8 years. It appears in different forms, as a little motile needle organism, as a globule or plasmodium, and agglutinates by cholera serum.

In the bacteriologic literature we find much about "mutation;" most authors, however, do not mean new constant varieties, but only a sudden change, e. g., the unusual appearance of the colonies, the faculty to ferment a new kind of sugar, etc. In my opinion the observations on the asporogenous *B. anthracis* have more interest with regard to mutation. As far as I know, no one has continued these experiments long enough. E. C. Hansen studied asporogenous *saccharomyces* varieties for sixteen years and proved their constancy.

In 1910, Herzog¹⁵ suggested that the bacteria of trachoma descend from *Micrococcus gonorrhea*. A large coccus was said to be transformed into a very small one, and the new form was found to be able to live and multiply as such.¹⁶ It will be interesting to learn whether the small cocci are able to reproduce the larger forms.

Beyond doubt several pathogenic strains originate from other bacterial species. Primarily I think of the coccus of pemphigus and impetigo contagiosa, *M. pemphigi-neonatorum*.¹⁷ We find many cocci in the vesicles. They are easy to isolate by plating and agree almost completely with *M. pyogenes-aureus*. The difference is pathologic; one coccus produces limpid vesicles in the skin, the other pus. I discovered this in 1890, and for almost thirty years we have continued these observations.¹⁸ In the United States the same results have been obtained by Clegg and Wherry¹⁹ and by Falls.¹⁹

¹⁵ Ueber die Natur und Herkunft des Trachomerregers, 1910.

¹⁶ Ibid., p. 46.

¹⁷ Almquist, E.: Pemphigus neonatorum, etc., Ztschr. f. Hyg. u. Infektionskr., 1891, 10. u. 253.

¹⁸ The Etiology of Pemphigus Contagiosus, Jour. Infect. Dis., 1906, 3, p. 165.

¹⁹ The Bacteriology of Pemphigus Neonatorum, ibid., 1917, 20, p. 86.

It is remarkable that granules from a bacterial species can be separated from the culture by filtering and are able to multiply as such. Some years ago Löhnis partially verified these observations by filtering saprophytic bacteria. He writes: "Gonidia grow to new cells very readily while still connected with the parent cell, or if they have otherwise access to genuine plasma, like that in dead or dying cells of fungi and algae. But in other cases free gonidia have rarely been found to be inclined to act promptly as normal reproductive organs when kept under ordinary laboratory conditions."²⁰ The isolated granules do not grow readily. By making use of substrates containing lactose, lactate, sterile algae or sterile fecal matter I succeeded. Now after eleven years the same strains are adapted also to agar and to a temperature of 37 C.

The second difficulty is to verify the granules. The agglutination and Pfeiffer's test were decisive for numerous strains. The similarity to the granules in the cultures of *B. typhosus* helps to confirm their identity, but errors may occur. Thus far I have published only my results about agglutinating strains, produced by heating, and *B. antityphosum* by filtering.¹⁴

It is remarkable that it is possible to separate a minute part of *B. typhosus*, which is able to multiply as such. Beyond doubt we stand here at the beginning of an analysis of a bacterium, that is to say, of a living organism in quite a new manner.

B. antityphosum also passes through a life cycle. It appears usually as minute ovals, grows larger on sugar substrates, produces a small branched plasmodium as a promycelium, thrives well at 10 C. and competes there successfully with *B. typhosus*; on certain substrates it grows in thick layers. In comparison with the long, motile parent cells it is much reduced. We must expect to find similar minute forms in our environment. Probably most of them are difficult to cultivate.

It is obvious that these minute forms must be necessary for the parent strains, though they also may have their own existence. We believe that they readily fuse with other forms of the same strain and cause a kind of synthesis and it seems not impossible that a synthesis takes place also with foreign forms. In the symplastic stage we must expect surprises of various kinds. The bacterial synthesis and analysis are of great importance to biology.

²⁰ Footnote 2, p. 183.

IMPORTANCE OF VARIATION

The variation of the fungi is manifold; even the same species may produce many different spores. The bacteria perhaps vary still more. We already know true series and different generative bodies of many species.

It must be borne in mind that all variation helps the species itself. In old cultures we often find a periodicity of growth; after an interval secondary colonies appear, and after another interval perhaps tertiary colonies, according to the ability of the strain to adapt itself to new conditions. For variation and adaptation the plasmodium and the whole life cycle are essential. Löhnis suggests that the bacteria in the symplastic stage are in a much better position to adapt themselves to changed environmental conditions than they are during their cellular life.²¹

Perty has referred to the plasmodium as a very fine "Punktsubstanz." He saw the cells dissolve and form the plasmodium, which was transformed into new organisms of the lowest kind.²²

For the pathogenic bacteria a low temperature and a drying substrate are of great importance in producing plasmodia and regenerative bodies. A kind of rejuvenation takes place often after passing the life cycle. The new forms often exhibit great constancy, for instance, in the loss of motility, in their power to produce conidia, etc.

Increased virulence is another rather common occurrence when pathogenic bacteria are grown saprophytically. Koraen found first that some strains of *B. typhosus* when grown at low temperature gain resistance against agglutinating serum and the lytic power of the blood. We also found that the virulence can be increased when *B. typhosus* is cultivated for a long time. Olsson discovered that *Spirillum cholerae* became more virulent after having passed a long series of development.¹¹ *B. diphtheriae* is able to recover its virulence after it has passed the plasmodium stage. The same was observed with an avirulent degenerated strain.

The common method for augmenting the virulence is the "passages" from animal to animal, but this method cannot claim general validity. In some epidemics we observe that the "passages" from man to man diminish the virulence and at last extinguish it completely, e. g., in measles. In the saprophytic growth many bacteria regain their pathogenicity; probably this is quite common with many species.

²¹ Footnote 2, p. 196.

²² Footnote 6, p. 113.

Pathogenicity.—The textbooks often know more than the research workers. We read that the common rods of *B. typhosus* pass through the intestines, rest in the glands, etc. However, who has studied the forms of *B. typhosus* in the living human body? The big oval forms in the dead human body are well known, but still their origin is in doubt. Perhaps they constitute an intermediate stage between plasmodium and rod.

In the living blood of typhoid patients I observed, in 1881, in Ranvier's moist chamber before coagulation took place: (1) common rods with their characteristic motility—rare, but sometimes in abundance; (2) a little immotile needle, fairly common; (3) rather big oval forms, rare. All three forms took the stain easily. After a few days I discovered in my hanging drops occasional granules germinating to a kind of fine plasmodium, similar to the plasmodium of *B. antityphosum*.²³ The phenomenon was not common, but in one case I found 20 germinating granules in the same drop. These observations should be continued.

Within a week the common rods of *B. typhosus*, *B. dysenteriae* and *Spirillum cholerae* may be transformed by the saprophytic growth. They are then able to produce plasmodia and conidia. In the human body each rod of *B. typhosus* and *B. dysenteriae* may produce a number of the smallest forms which easily pass the intestinal wall. The minute size and their great number make the passage easier for them than for the rods. Perhaps the plasmodium and the granules constitute the chief forms within the sick body.

Epidemiology.—A chiefly mechanical theory is insufficient to explain epidemics. Season, meteorology, etc., are important factors in the appearance of many epidemics, but most authors pay little attention to the relation between seasons and epidemics, and to the causes of the difference in the virulence of the strains.

The study of the different stages in the development of the bacteria will make it possible to explain both the pathogenicity and the rise and decline of many epidemics. Plasmodia and fructification facilitate immensely the invasion of new individuals. Furthermore, the saprophytic growth restores the virulence, e. g., of *B. diphtheriae*. It is well known that new cases of diphtheria in the same family often occur after one or two months. This fact may be explained by the increased

²³ Footnote 4, second part, plates 14 and 15.

virulence, since the bacteria have passed the stage of plasmodium. On the other hand, minute, avirulent forms, similar to *B. antityphosum*, possibly help to make a population immune.

Classification.—In 1899 Willibald Winkler²⁴ of Vienna pointed out the great importance of the plasmodia that are able to produce bacteria, "Teilungsplasmodia" and sporangia. Probably all colonies and the so-called zooglea develop from plasmodia. The branched forms of *B. tuberculosis*, *B. leprae*, *B. diphtheriae*, etc., are connected with the plasmodial stage. The bacteria are declared to be related to the myxomycetes. These important discoveries and theories will probably sometime be regarded as the starting point for a natural system of bacteria. Thus far the authors have been too much occupied with subjective essays of ephemeral nature on bacterial classification.

TERMINOLOGY

For creating standard terms our knowledge is too incomplete. We must wait until both the bacteria and the other classes of fungi have been investigated more thoroughly. At present the chief endeavor is to observe and describe exactly, and to use appropriate terms.

In my earlier papers I pointed out the temporary value of my own terms. I looked for analogies in the mycology of DeBary and of Brefeld and added bacterial to each term. Thus I speak of bacterial plasmodia, bacterial conidia, etc., because I cannot decide whether my findings agree with those concerning plasmodia, etc., in other parts of the mycology.

Bacterial mutation.—Formation of a new constant variety or species. Except hybrids, probably all new species are produced by mutation.

Bacterial plasmodium.—Protoplasmatic clump, the amorphous stage of the species (Figs. e and q). Löhnis calls it symplasm. It is somewhat similar to the promycelium of Tulasne.

Conjunction consists of the direct union of two or more individual cells.²⁵ Perty has spoken of this; copulation is a similar phenomenon—a creation of new individuals.²⁶

Myceloid.—Long, intricate threads without branches

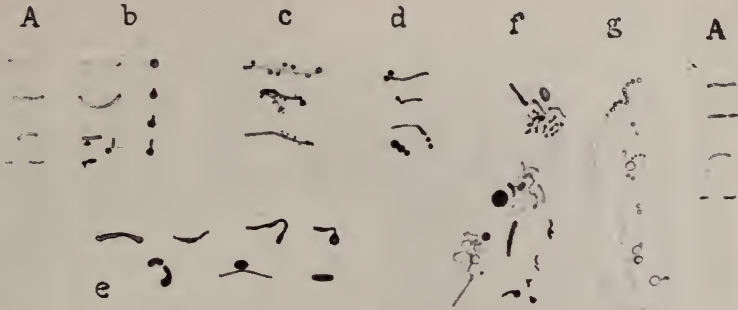
Bacterial conidium.—Exogenous globule sprouting to a rod, spirillum or a new globule (Figs. b to f and i to m); Löhnis calls it regenerative body or exospore.

²⁴ Untersuchungen über das Wesen der Bakterien, Centralbl. f. Bakteriöl., ii, 1899, 5, p. 569.

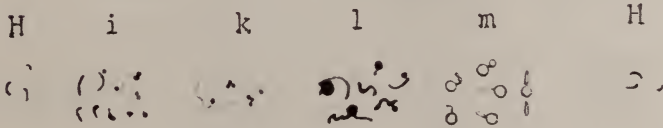
²⁵ Footnote 2, p. 197.

²⁶ Footnote 6, p. 220.

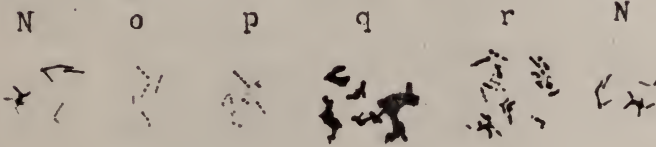
B. typhosus



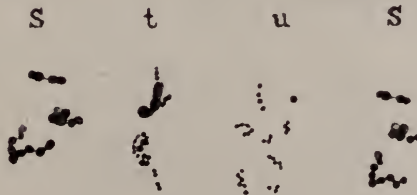
Spirillum cholerae



B. diphtheriae



Microc. Thulini



The figures are drawn from photographs; only figs. *g* and *m* were made directly from the microscopic observation; $\times 1,000$. *B. typhosus*: A, rods of different size; b to e, their development on drying substrate at 10-14 C.; b on agar for one week; thick rods with lateral and terminal conidia, free conidia germinating; c, lateral microconidia; d, after 4 weeks on agar; e, after 3 weeks on potato, plasmodia; f, transformed cultures germinating on serum at 37 C.; three sporangia are open, their content is made up of finest curved chains and globules; g, germination directly observed under the microscope. *Spirillum cholerae*: H, common spirilla; i, the same after a few days at 15 C.; longer and larger forms, plasmodia, conidia, some germinating; k, on drying agar at 14 C. after one month; empty threads, conidia; l, transformed culture 4 hours at 37 C.; thread and germinating conidia; m, the same culture 4 hours at 22 C.; conidia germinating to spirilla and globules. *B. diphtheriae*: N, Löffler's rod; o, p, on lactate substrate 2 to 3 weeks at 19 or 25 C.; similar to small oida; q, in drying broth at 37 C. after one month; big clumps—plasmodia; r, the clumps turning to rods. *Micrococcus Thulini*: S, great cocci similar to staphylococci; t, the great cocci after 5 days begin to produce small cocci, similar to streptococci; u, in broth at 35 C. after 2 weeks; fine cocci only, on agar large cocci are reproduced.

Bacterial microconidium.—A very small exogenous granule with a diameter of about $\frac{1}{2}$ micron (Fig. c). Löhnis uses the term gonidium.

Bacterial sporangium.—Large exogenous conidium, containing several cells, globules or chains (Figs. f and g). Löhnis prefers the term gonidangium.

Bacterial oidium is produced by the segmentation of a rod or thread (Figs. o and p). In 1890, I introduced the term oidium for the spores of *Streptothrix* or *Actinomyces*.²⁷ The type is *Oidium lactis*. This spore is also named arthrospore.

Bacterial micro-oidium is a very small oidium, difficult to observe with the microscope, better visible in the photographs (Figs. f and g).

²⁷ Untersuchungen über einige Bacteriengattungen mit Mycelien, Ztschr. f. Hyg. u. Infektionskr., 1890, 8, p. 189.

A NEW METHOD FOR DIFFERENTIAL STAINING OF BACTERIA

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In 1884, Christian Gram¹ while working with sections of nephritic material, discovered that if he stained the tissue with Ehrlich's anilin gentian violet solution and then with Lugol's solution a subsequent treatment with alcohol decolorized the tissue but did not remove the stain from some pathogenic bacteria in it. Further tests by Gram and others showed that the procedure could be used to divide the family of bacteria into two great groups: those that retained the stain and those that were decolorized when treated with alcohol. The method proposed by him has been closely followed in theory ever since and has gained universal application in bacteriology on account of the diagnostic value of the data obtained from it. The changes in method that have been suggested from time to time have been confined, for the most part, to various ways of preparing the violet solution in order to obtain one of better keeping quality. Recently Burke² has described a method for the Gram stain in which he has recommended the use of acetone or acetone and ether for the decolorizer.

The procedure reported here, while differentiating the same groups of organisms as Gram's method, does not follow the theory of his work, since, along with other differences, the iodine solution is not used.

METHOD

Solutions: The stain used is cotton blue C₄B (Poirrier's Blue)³ which is obtained by the action of sulphuric acid on triphenylrosanilin.

Received for publication, Aug. 14, 1922.

¹ Fortschritte der Medicin, 1884, 2, p. 185.

² Jour. Bacteriol., 1922, 7, p. 159.

³ The cotton blue C₄B may be obtained from Walter F. Sykes & Company, 8 Lispenard Street, New York. This firm is the American representative of Societe Anonyme des Matieres Colorantes et Produits Chimiques de Saint-Denis.

One sample of an American made cotton blue C₄B was tested but did not give satisfactory results. The dye became a reddish purple in some of the positive cultures after counter-staining, and when a small amount of acid was added to the solution to make the blue persist the negative cultures did not decolorize properly. It did not seem worth while to work further with the American dye at present because no doubt the process for making it will be improved so that a dye comparable to that originally patented by Poirrier will be produced. In this connection, it is interesting to note that the present imported sample is exactly the same in reaction as a nine-year old one with which this work was started.

One gram of the dye is placed in a mortar, moistened with 1 cc of 95% alcohol and then stirred and ground with a glass stirring rod for a couple of minutes, until the mixture becomes a thick, smooth liquid. One hundred cc of an aqueous phenol solution are added and the grinding continued for a minute or two until all lumps of dye have dissolved, when the solution is ready for use. The phenol solution is prepared by adding 5 cc of the melted phenol crystals to 100 cc of distilled water, shaking vigorously and then filtering.

The second solution is a combined decolorizing and counterstaining agent. It is prepared by dissolving 2 gm. of safranin⁴ in 100 cc of 95% alcohol and then adding 100 cc of acetone c. p.² The alcoholic soluble safranin was used but a test with a water soluble sample gave equally good results.

Procedure.—The bacterial film is spread in the usual way. The customary precaution should be taken not to make it too heavy. If allowed to dry on the slide no fixing is necessary, but the drying and fixing may be hastened by slightly heating if desired. The film is then covered with a few drops of the blue solution for from 20 to 30 seconds. The excess stain is drained off and the slide well washed in running water. The stain from the slide may be saved and used repeatedly. A couple of drops of the safranin solution are allowed to run down the washed slide to remove the excess water and the preparation then covered with the safranin solution for 3 or 4 minutes. The slide can be set down during this time as it is not necessary to tilt it back and forth to obtain a clearing of negative organisms. It is better practice to decolorize and counterstain in a Coplin jar.⁵ The slide may now be washed in running water and when dry is ready for examination.

The gram-positive organisms are colored a very deep blue and the negative ones the red characteristic of safranin.

Two species of micrococci tested with this method gave some blue cells and the rest dark purple when decolorized and counterstained for 3 minutes. In order to obtain a larger number showing characteristic blue staining the cotton blue C₄B solution was left on the film for 1 minute and the decolorizing and counterstaining continued for 4 minutes. This change gave the desired result and a smear of *B. coli* on the same slide was stained red.

RESULTS

The procedure outlined was used in a test on 16 cultures of bacteria that had also been stained with Ehrlich's anilin gentian violet solution in the usual way for Gram staining. In every instance the proposed method gave the same positive and negative results that were obtained with the anilin gentian violet stain.

The organisms⁶ employed for this work were:

⁴ Safranins obtained from Sykes & Company, Coleman and Bell (safranin A conc.), Shoemaker and Busch, and the Will Corporation gave satisfactory results.

⁵ On a very warm day during which there was considerable evaporation the acetone-alcohol solution volatilized so fast that a slide became almost dry and the solution so concentrated that the positive organisms were decolorized and counterstained. This was the only time, however, when the usual method of applying the stain failed to give satisfactory results. A duplicate slide placed in a Coplin jar was properly decolorized and counterstained at this time.

⁶ For some of these cultures the author is indebted to Messrs. N. R. Smith and L. T. Leonard of this office, to Dr. J. M. Sherman of the Dairy Division, Bureau of Animal Industry, U. S. D. A., and to Dr. E. Lefevre, Microbiological Laboratory, Bureau of Chemistry, U. S. D. A.

Positive	Negative
<i>B. asterosporus</i>	<i>B. coli</i>
<i>B. cereus</i>	<i>B. fluorescens</i>
<i>B. megatherium</i>	<i>B. prodigiosus</i>
<i>B. simplex</i>	<i>B. radicolica</i> (red clover)
<i>B. subtilis</i>	<i>B. radicolica</i> (vetch)
<i>B. vulgatus</i>	<i>Ps. pyocyanea</i>
<i>Bact. mycoides</i>	
<i>M. pyogenes</i> var. <i>aureus</i>	
<i>M. candidus</i>	
<i>Mycobact. diphtheriae</i>	

THE THEORY

The phenomenon associated with the retention of the violet stain in the Gram method has been explained as due to purely chemical reactions by Unna⁷ and to physical ones by Brudny.⁸ Benians,⁹ in a series of experiments to test the basis of these explanations, while not wholly agreeing with Brudny, does come to the conclusion that the reaction is a physical one. Previous investigators in seeking a solution of this problem have been under the disadvantage of having to precipitate the violet stain within the cell before they could test for decolorization. In the procedure presented here that difficulty is removed, and so the explanation is to that extent simplified.

The imported cotton blue C_4B when made up in aqueous solution and employed in the usual way is retained¹⁰ by a few cells in a positive culture although the great majority when counterstained are red or purple. When the stain is made up in the phenol solution all the cells are blue after counterstaining. The stain is soluble in phenol, which, possessing a great affinity for living matter, thus fixes the dye to the cell substance. This fixing power is due to a physicochemical reaction.

The data obtained indicate that physical phenomena control the results in the remainder of the procedure. If 1.5 gm. of boric acid¹¹ are dissolved in 100 c c of the decolorizing and counterstaining reagent, to increase the solution pressure, and films of different cultures stained with cotton blue C_4B , then treated with it for 6 minutes, a large number of cells in certain weakly gram-positive cultures show a negative staining

⁷ Monatschr. f. prakt. Dermat., Ergänzungsheft, 1887.

⁸ Centrallbl. f. Bakteriöl. u. Parasitenk., II, 1908, 21, p. 62.

⁹ Jour. Path. & Bacteriol., 1912-13, 17, p. 199; 1920, 23, p. 401.

¹⁰ In the usual Gram staining method this is recognized as a characteristic of dyes of the pararosanilin group.

¹¹ Sodium acetate or ammonium nitrate will give the same result.

while films of the same cultures, if dried on a double filter paper for 1 minute before immersion in the decolorizing and counterstaining solution, show practically all positive cells. The strongly positive species remain blue under both treatments. Further tests may show that a definite grouping of strongly and weakly positive species may be obtained by a comparison of results on moist and dried slides with this decolorizing and counterstaining solution containing boric acid. *B. coli* is negative, except possibly for a blue ring around the edge of the smear where it is thickest, under this treatment when decolorized and counterstained for 6 minutes; but if the time of decolorization is limited to 3 minutes, the moist films are negative and the dried ones positive.

Strongly positive species may be made negative by covering a stained film of such a culture with the regular decolorizing and counterstaining solution and then gently heating on a hot plate for 5 minutes. A somewhat less complete decolorization and counterstaining may be obtained by immersing in the regular safranin solution to which 8 gm. of ammonium nitrate per 100 c.c. have been added. At the end of 2 hours the slide is placed in distilled water for 20 minutes and then returned to the solution mentioned in the foregoing for about 3 hours, when it is placed in the safranin solution containing boric acid for a short time. This procedure yields a majority of pale pink rods with some pinkish purple and some blue ones.

Acetone acts as a dehydrating agent only, and by removing the water, as drying with filter paper does, yields a larger number of blue cells in weakly positive cultures. If the decolorizing and counterstaining solution contains only 25% or less of acetone, more red and purple cells are found in such cultures, showing that the dehydration has not been sufficient to reduce the speed of osmosis.

All these facts indicate that the phenomenon depends on the permeability of the cell sheath and that as the water within the cell is reduced osmosis is correspondingly slowed up so that a much longer time is required to decolorize it.

DISCUSSION

The addition of a small amount of alcohol is advised for the preparation of the solution of cotton blue C_4B , because some of the stain dissolves in it and the remainder in this case goes into solution more readily in the phenol water. The solution prepared in this way seems to keep indefinitely, as one made eight months ago shows no change and stains as well now as when first tried. The only precaution taken to preserve it was to keep the bottle tightly stoppered.

It may not be amiss here to call attention to the fact that safranin is better for this procedure than other red dyes because it does not overstain if left on a smear longer than usual.

It hardly seems necessary to point out the advantages of a procedure which entirely eliminates the use of one solution that has always been considered necessary, and which also provides for simultaneous decolorizing and counterstaining of bacterial films. This method also gives the bacteriologist an opportunity to adopt a uniform time for decolorizing preparations.

The organisms used in this procedure were stained when they were 1 and also 3 days old. The results were the same in both instances.

The slides showing the greatest contrast were obtained by staining and decolorizing for the longer periods, namely, 30 seconds and 4 minutes. Some of the species stained with the blue for 5 to 10 seconds and then decolorized and counterstained for 45 to 60 seconds showed characteristic coloration; but since other species gave better results with slightly longer staining, the longer time is recommended.

SUMMARY

A new method is presented for the differential staining of bacteria. It yields results like those obtained with Gram's procedure.

A cotton blue C₄B solution is employed for the initial staining.

No iodine is used.

Decolorizing and counterstaining are done with one solution.

When tested with a decolorizing and counterstaining solution containing boric acid, sodium acetate or ammonium nitrate, a large number of cells in weakly gram-positive cultures were negative; but when a film of the same culture was dried on filter paper nearly all the cells were strongly positive.

A film of *B. coli* was positive on a dried slide that was decolorized and counterstained (solution containing boric acid) for the same length of time that gave a negative staining on a moist one.

Species that are strongly positive become negative when the film of such a culture covered with the regular decolorizing and counterstaining reagent is gently heated for five minutes. A less complete decolorization and counterstaining may be obtained by immersing for a long time in the regular solution to which an excess of ammonium nitrate has been added.

The reaction is a physicochemical one depending on the affinity of the dye for cell substance, supplemented by the fixing power of phenol, and the permeability of the cell sheath.

ISOLATION OF ANTHRAX BACILLUS FROM A SHAVING MUG

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During the last few years many cases of anthrax have occurred in man from the use of shaving brushes contaminated with anthrax bacilli.

Lederer and Leak¹ state that "due to the mobilization of large forces in the warring nations the demand for shaving brushes soon exceeded the supply, and bristles from questionable sources were employed in their manufacture and outbreaks of anthrax reported followed as a logical consequence. Among English troops in France 28 cases occurred from 1915 to February, 1917. Eighteen infections with anthrax occurred among the troops in England from the beginning of the war until February, 1917. The most thoroughly studied series, however, was that of 19 civilian cases in Great Britain. In most of these cases the proof of the source of infection was absolute, and in other cases circumstantial evidence was strong."

Norton and Kohman² report a fatal case in a soldier following the use of a shaving brush recently purchased by the patient and which contained anthrax bacilli. Symmers and Cady³ examined 40 shaving brushes purchased from peddlers in New York and from shops, and 3 were found to harbor virulent anthrax bacilli. Casey⁴ found that the isolation of anthrax bacilli from shaving brushes is accomplished better by animal inoculation than by culture.

REPORT OF CASE OF ANTHRAX

On May 31, 1922, a patient with anthrax of the neck was admitted to the Johns Hopkins Hospital, and the health department was called on for anthrax serum, which was given; on June 6, the patient was convalescent. The anthrax bacillus had been isolated from the pustules in the laboratory of the hospital, but no organisms were isolated from the shaving brush used by the patient. By mistake the brush was boiled for 2 hours in the hospital laboratory. The shaving mug had not been obtained. The shaving mug previously used by the patient was sent to the health department laboratory for examination. This cup had been on the market for 2 years and was found to contain virulent anthrax bacilli.

The cup itself was stuffed with paper, which was soaked in sterile salt solution for 2 hours; the cup was washed thoroughly in salt solu-

Received for publication Aug. 17, 1922.

¹ Am. Jour. Pub. Health, 1919, 9, p. 114.

² Jour. Am. Med. Assn., 1919, 72, p. 1729.

³ Ibid., 1921, 77, p. 212.

⁴ Am. Jour. Med. Sc., 1920, p. 742.

tion also. The cup washings and the solution in which the paper soaked were mixed and centrifugated for 5 minutes. The supernatant fluid was poured off and the sediment mixed with salt solution, about 4 c c of which were inoculated subcutaneously into a guinea-pig, which died in 72 hours with characteristic lesions. Smears from the liver, spleen, and heart blood showed a gram-positive bacillus and cultures were made on agar slants and plates. After 24 hours, the agar tubes showed pure cultures of anthrax bacilli, which again produced the disease in a guinea-pig. Paraffin sections showed the liver, spleen, and heart blood full of anthrax bacilli.

The remainder of the mug washings was inoculated on agar plates in 8 different dilutions and incubated for 48 hours, when colonies of anthrax bacilli were found to have developed.

In order to be absolutely sure of the diagnosis a loopful of the pure cultures was rubbed intracutaneously on the abdomen of a guinea-pig, which died in 44 hours. There was edema at the point of inoculation, and all organs showed typical anthrax characteristics, the spleen was greatly enlarged, and smears from the liver, spleen, and heart blood showed bamboo rod forms of anthrax bacilli surrounded by a capsule.

SUMMARY

From the results of the examination of washings of the cup it is evident that the shaving brush in all probability harbored anthrax bacilli, and that spores or bacilli adhered to the mug after the removal of the brush, showing again the great resistance of this organism.

AN EXPERIMENTAL STUDY OF THE METHODS
AVAILABLE FOR THE ENRICHMENT, DEMON-
STRATION AND ISOLATION OF *B. BOTU-*
LINUS IN SPECIMENS OF SOIL AND
ITS PRODUCTS, IN SUSPECTED FOOD,
IN CLINICAL AND IN NECROPSY
MATERIAL. I.*

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The bacteriology of botulism has, up to the present time, received scanty attention, because the investigation of the subject is fraught with technical difficulties and because the physiologic or experimental study of the disease offers more dramatic and obvious results. According to Burke,¹ 12 pure cultures of *B. botulinus* were available in 1919, in the United States; while, in 1921, Weiss,² in his study on the heat resistance of the spores of this organism, was able to use 14 to 16 toxin producing strains. In a recent communication Reddish³ reported on 19 strains of *B. botulinus*, of which 18 were found to be contaminated with *B. sporogenes*. Bitter,⁴ however, stated, in 1921, that in comparison to the fairly frequent occurrence of botulism in Europe, *B. botulinus* had been cultivated in a relatively small percentage of instances; namely, 12 isolations had been accomplished from food products, necropsy material or the feces of a hog. Most of these strains have been lost or, from the experience of one of the writers (K. F. M.), are so badly contaminated that they are useless for bacteriologic study. The conclusion of Bitter, that the demonstration and the isolation of *B. botulinus* from suspected food continues to be a comparatively difficult task, finds some justification in the fact that only 13, or 19%, of the 68 German botulism outbreaks have been bacteriologically proved. On the other hand, the studies of Randall,⁵ Nevin,⁶ Dickson,⁷ Burke,⁸ Thom, Edmonson and Giltner,⁹ Armstrong, Story and Scott,¹⁰ Graham¹¹ and others in this country furnish sufficient evidence that this anaerobe can be readily detected and purified from spoiled food material, provided suitable procedures and specimens are examined. Thirty-five, or

Received for publication, July 26, 1922.

* This study was aided by grants from the National Canners Association, the Canners League of California and the California Olive Association.

¹ Jour. Bacteriol., 1919, 4, p. 555.

² Jour. Infect. Dis., 1921, 28, p. 70.

³ Ibid., 1921, 29, p. 120.

⁴ Ergebn. d. allg. Path. u. path. Anat., 1921, 19, II, p. 762.

⁵ Med. Rec., 1920, 98, p. 763.

⁶ Jour. Infect. Dis., 1921, ~~33~~ p. 226.

⁷ Monograph of Rockefeller Institute, No. 8, 1918, p. 29; Jour. Am. Med. Assn., 1918, 71, p. 518.

⁸ Jour. Bacteriol., 1919, 4, p. 541.

⁹ Jour. Am. Med. Assn., 1919, 73, p. 907.

¹⁰ Public Health Repts., 1919, 34, p. 2877.

¹¹ Jour. Bacteriol., 1919, 4, p. 1. McCaskey: Am. Jour. Med. Sc., 1919, 158, p. 57.

34.6%, of the 101 recorded outbreaks have been proved bacteriologically or toxicologically. These differences in the percentages of isolations reported for Europe and the United States are probably not due to the superior technic employed in the latter country, but to peculiarities in the food products causing the intoxications. The isolation from spoiled food is probably simplified on account of the enrichment of the toxicogenic microbe and the relative scarcity of other bacteria. This scarcity may be the result of the selective action of the heat employed in the preservation of the products. It is known that in Germany only 2, while in the United States 78, outbreaks were caused by food preserved by heat.

A perusal of the publications dealing with the attempts to isolate *B. botulinus* from vegetables, manure and soil specimens, which contained few spores of *B. botulinus* and a mixed flora consisting of sporulating aerobes and anaerobes, clearly demonstrates that the customary methods employed hitherto have proved inadequate for dealing with numbers of strains of the organism. This is illustrated by the failures of van Ermengem¹² and Bitter¹³ to demonstrate *B. botulinus* in feces of animals and fishes and by the recent observations of Burke.⁸ The latter worker made 235 cultures from specimens consisting of tap water, hay, leaves, vegetables, soil, etc.; in 11, or 5%, of the cultures the toxin of *B. botulinus* was demonstrated, but the anaerobe was only isolated in a pure state from 2 cultures.

The collection of a large number of strains of *B. botulinus* from diverse sources suitable for a comparative bacteriologic and serologic study could only be attempted when the demonstration and isolation of the organism from bacterial mixtures was attended with less difficulty. The study of the distribution and habitat of *B. botulinus* in nature could not be approached with any degree of confidence until a practical method of examination had been elaborated. Although the problem appeared rather complex, sufficient information was available from previous work and from the intensive studies on anaerobes conducted during and immediately after the war to suggest some methods of attack. It is the purpose of this communication to outline the experiments which led to the use of suitable enrichment cultures and to procedures which made possible the isolation of a fair percentage of pure strains of *B. botulinus*.

EXPERIMENTAL DATA

It is well known that *B. botulinus* can best be recognized and differentiated from other anaerobes by the action of its toxin on small laboratory animals. Repeated observations have shown that the poison may develop in mixtures, notwithstanding the presence of other aerobic or anaerobic bacteria. This latter property can therefore be used for the detection of *B. botulinus*. It is equally true that a medium encouraging the toxin production will also lead to an enrichment of *B. botulinus*.

¹² Handb. d. pathogen. Mikroorganism, 1912, 4, p. 936.

¹³ Reference 4, p. 791.

However, when the studies on which this paper is based were undertaken, the biochemical requirements of a culture fluid necessary to toxin production were poorly understood. In fact, some of the published statements were contradictory; a few workers preferred an alkaline reaction, while others selected a neutral culture fluid made of pork. The period of incubation, the temperature and oxygen requirements, etc., essential for the maximum toxin production had received some attention, but the published experiences were frequently not confirmed, and one gained the impression that many of the results depended more on chance than on reliable procedure. The experiments planned to elaborate a practical method for the enrichment and subsequent isolation of *B. botulinus* in pure culture had therefore to taken into consideration the following subjects: (1) composition and reaction of the culture mediums; (2) methods of anaerobiosis; (3) precautions against contaminations; (4) amount and preparation of field material to be examined; (5) period of incubation; (6) storage of cultures; (7) identification of toxin; (8) enrichment of weekly toxic cultures.

COMPOSITION AND REACTION OF CULTURE MEDIUMS

The original observations made by van Ermengem¹⁴ that *B. botulinus* grew abundantly and produced a potent toxin in double strength, slightly alkaline meat infusion broth containing 1% peptone, 1% sodium chloride and 2% glucose have until recently been responsible for the use of this medium when selecting a substratum for the isolation of the organism from food or field specimens. Some workers adhered faithfully to the formula and employed a pork infusion broth of a reaction of 0.5% alkaline to phenolphthalein. It has already been pointed out that Dickson and Burke, Graham and others succeeded in isolating *B. botulinus* with the aid of this medium. The successes can probably be attributed to one or two causes: (1) cultures made of the samples contained a large number of spores of *B. botulinus*, and (2) a relatively small number of concomitant anaerobic spore bearers. Theoretically the medium of van Ermengem, although excellent for the toxin production with pure strains of *B. botulinus*, is, on account of its high carbohydrate content and relatively low buffer value, not selective for proteolytic anaerobes. Biochemical studies indicate that the van Ermengem broth favors the saccharolytic anaerobes and, when inoculated with a mixture of anaerobes, reaches frequently an H-ion concentration which is inhibitive for *B. botulinus* or at least for the enzymes

¹⁴ Ztschr. f. Hyg. u. Infektionskrankh., 1897, 26, p. 1.

assumed to be responsible for the liberation of the specific toxin. Furthermore, it has been noted that sporulation of *B. botulinus* is usually poor in van Ermengem mediums, as a result of which the isolation of this organism by selective heating of the enrichment culture is impossible. Chemical studies have shown that a pork base offers no advantages over a veal or beef infusion. In a search for a suitable medium, it was fully realized that solid mediums in the form of blood-agar plates or shake cultures in deep agar were useless on account of the comparative scarcity of *B. botulinus* spores in most of the specimens studied in the beginning of this investigation. The ideal medium for the isolation of *B. botulinus* would therefore be a fluid medium which favored the growth of that organism and restrained completely the development of the other anaerobic and aerobic bacteria commonly present in the soil and its products. The same considerations led Tulloch,¹⁵ in 1917, to prepare a medium that provided enrichment of *B. tetani* in cultures made from wounds containing a variety of organisms. This worker noted that a substratum in which *B. sporogenes* had grown for some time was not suitable for the growth of the saccharolytic anaerobes or of *B. sporogenes* itself. Miss F. Field, of this laboratory tried, in tedious and time consuming experiments, to develop "exhaust mediums" suitable for *B. botulinus*. In these experiments, meat and vegetable substratums were inoculated with *B. sporogenes*, soil anaerobes, etc., and incubated for varying periods of time in open and closed vessels, but no medium thus prepared showed consistently selective properties for *B. botulinus*. Attempts were also made to enhance the selective properties of the exhaust mediums by the addition of pieces of string beans, glycerol, starch, etc., but the results were unsatisfactory. In fact, the observations collected in the course of these studies indicated that a completely exhausted medium for *B. sporogenes* could not be obtained and that a fluid medium partially inhibitive for *B. sporogenes* invariably exerted the same influence on *B. botulinus*. Furthermore, the preparation of exhaust mediums is time consuming and expensive when used for the enrichment of soil specimens weighing from 50 to 100 gm. These conclusions influence in no way the value of the exhaust medium of Tulloch for the isolation of *B. tetani*. A comparative study, conducted by one of us (K. F. M.), in 1918, confirmed the facts established by this worker. However, one must appreciate that the bacterial flora of a wound infected with the

¹⁵ Jour. Roy. Army Med. Corps, London, 1917, 29, p. 631.

spores of the tetanus bacillus is less complex than that ordinarily encountered in specimens submitted to a search for *B. botulinus*. It is not unlikely that the addition of a piece of rabbit kidney enhances the growth promoting properties of the exhaust medium for *B. tetani*, which has already been selectively enriched in the wound secretion. The experience of this laboratory, however, indicates that the exhaust medium of Tulloch loses its selective property in the isolation of *B. tetani* from garden soil or other specimens containing a rich flora of anaerobic bacteria. Similar observations probably prompted Adamson¹⁶ to recommend an alkaline broth, instead of an "exhaust medium" for the isolation of this particular organism.

In the preliminary investigations, it was noted that the elaboration of toxin by impure cultures of *B. botulinus* was excellent in a beef heart peptic digest medium¹⁷ employed for the studies of anaerobes in this laboratory. Subsequent biochemical studies to be published elsewhere have confirmed these observations and furnished accurate data explaining fully the superior properties of this medium over the others commonly used in the study of anaerobes. Furthermore, it was determined that the medium of van Ermengem could be improved by reducing the amount of glucose from 2 to 1%, by adding 0.5% dibasic potassium phosphate, only 0.5% sodium chloride and by adjusting the final reaction to P_H 8.4. This medium buffers excellently the volatile and fixed acids produced by the anaerobes; thus the H-ion concentration rarely increases over a P_H of 7 to 7.2. In fact, the reaction remains at the optimum H-ion concentration of the growth curve of *B. botulinus*, as will be shown by C. C. Dozier, in another paper. It is advisable either to add the necessary amount of glucose in the form of a 25%

¹⁶ Jour. Path. & Bacteriol., 1920, 23, p. 241.

¹⁷ Preparation of beef heart peptic digest liver broth (for principles involved see Holman: Jour. Bacteriol., 1919, 4, p. 149; Stickel and Meyer: Jour. Infect. Dis., 1918, 23, p. 68, and H. H. Heller: Jour. Bacteriol., 1921, 6, p. 445). (1) Slowly heat to boiling finely ground, fat free heart, 1,000 gm., and tap water, 1,000 c.c.; adjust to a reaction of P_H 8.0-8.2, then cool and carefully skim off the layer of fat which floats on the cold medium. To each liter of beef heart mash, add 2 liters of peptic digest broth (see 2). Adjust the reaction to P_H 7.2-7.4. (2) Wash clean and mince finely 5 or more large pigs' stomachs. Mince an equal amount of clean pig's or beef liver. Mix in the following proportions:

Minced pigs' stomachs.....	400 gm.
Minced liver.....	400 gm.
Hydrochloric acid (Baker Chemical Co.).....	40 gm.
Tap water at 50 C.....	4,000 gm.

Keep the mixture in glass or porcelain receptacles for 18-24 hours. Make biuret and also tryptophan test. When both reactions are positive, the digest is green-yellowish and contains little undigested debris. Transfer to large bottles and steam for 10 minutes at 100 C. to stop digestion. Strain the digest through cotton, or preferably store over night in the ice chest and decant after 24 hours. Warm the decanted digest to 70 C. and neutralize with sodium carbonate (twice normal solution) to litmus at this temperature. Filter the desired amount, add 0.2% dibasic potassium phosphate; adjust to P_H 7.4 and mix with beef heart mash. Adjust the final reactions and sterilize for one hour at 18 lbs. of pressure. Incubate for 5 days and repeat the same sterilization for one hour at 18 lbs. of pressure.

solution after sterilization of the alkaline broth in the autoclave or to adjust the reaction of the medium with sterile sodium carbonate solution shortly before use. Live steam sterilization in an Arnold steam sterilizer produces less change and a medium of superior properties, but for reasons to be discussed later, this method of sterilization should not be employed for anaerobes. The beef peptic digest liver broth can be autoclaved without inducing changes in the reaction or in the growth

TABLE 1
RESULTS OF EXPERIMENTS WITH SOIL AND VEGETABLE SPECIMENS

Number	Material	Medium	Incubation, Days	Result
3 A	Soil 5 gm.	van Ermengem....	22	Negative
3 B	Soil 5 gm.	Beef heart digest...	22	2 cc toxic for guinea-pigs in less than 24 hours
3 C	Bean stalks 20 gm.	van Ermengem....	22	Weak toxin, 2 cc toxic for guinea-pigs after 12 days
3 D	Bean stalks 20 gm.	Beef heart digest...	22	2 cc toxic for guinea-pigs in less than 24 hours, type A
3 E	Bean casing.....	van Ermengem....	22	2 cc toxic for guinea-pigs in 48 hours, type A
3 F	Bean casing.....	Beef heart.....	22	2 cc toxic for guinea-pigs in less than 24 hours, type A
10 II	Soil 10 gm.	van Ermengem....	28	2 cc nontoxic
10 Ia	Soil 10 gm.	Beef heart digest...	28	2 cc toxic for guinea-pigs in less than 24 hours, type A
10 Va	5 gm. decomposed hay....	van Ermengem....	28	2 cc nontoxic
10 Vb	5 gm. decomposed hay....	Beef heart digest...	28	2 cc toxic in 5 days
OK II	Turnips 20 gm.	van Ermengem....	34	Nontoxic
OK II	Turnips 20 gm.	Beef heart digest...	34	2 cc toxic for guinea-pigs in 5 days
OK III	Cherries 20 gm.	van Ermengem....	34	Nontoxic
OK III	Cherries 20 gm.	Beef heart digest...	34	2 cc toxic for guinea-pigs in 6 days
OK5 II	Leaf mold.....	van Ermengem....	25	2 cc toxic for guinea-pigs in 5 days
OK5 II	Leaf mold.....	Beef heart digest...	25	2 cc toxic for guinea-pigs in 3 days, type A
OK5 V	Asparagus tip.....	van Ermengem....	34	2 cc toxic for guinea-pigs in 6 days
OK5 V	Asparagus tip.....	Beef heart digest...	34	2 cc toxic for guinea-pigs in 2 days, type A
02K 9	Asparagus stalks.....	van Ermengem....	38	2 cc nontoxic
02K 9	Asparagus stalks.....	Beef heart.....	38	2 cc toxic for guinea-pigs in less than 16 hours, type B

supporting and enhancing properties of the enrichment mediums. The optimum reaction of this medium is a P_H of 7.0-7.4.

The value of the two mediums has been repeatedly tested; three experiments carried out early in the course of this investigation are, however, chosen for a discussion of the relative merit of the two mediums.

Exper. 1, 2 and 3.—Soil and vegetable specimens were suspended in salt solution heated for from 1 to 2 hours at 70 C. The heated suspensions were divided in equal portions and mixed in flasks with modified van Ermengem's broth (P_H 8.4) or beef heart peptic broth (P_H 7.4), exhausted and incubated for from 22 to 38 days. The results are summarized in table 1.

It is evident from the data presented in table 1 that the modified van Ermengem medium is decidedly inferior to the beef heart digest broth. Seven specimens contained a sufficiently large number of spores to produce in the latter medium a strong toxin, which could be identified by an antitoxin neutralization test, while only one glucose broth culture gave the same result. In fact, 6 cultures made in this medium were nontoxic and failed to indicate the presence of *B. botulinus* spores in the specimen. In 3 van Ermengem cultures the toxin produced was very weak and its nature could not be proved by the customary antitoxin test. Entirely similar results could be cited from other experiments.

Another great disadvantage of the van Ermengem broth is the violent gas production which takes place in soil cultures. A considerable number of culture flasks broke under the pressure of the liberated gases and valuable specimens were lost in this manner.

Repeated attempts to isolate *B. botulinus* in a pure state from positive enrichment cultures in van Ermengem's medium were either not successful or were in comparison with those made from beef heart mediums considerably more difficult. The principle of selective heating could not be applied to these cultures, probably on account of the absence of, or the low heat resistance of, the spores. These and other observations led to the routine use of a beef heart peptic digest liver broth for the enrichment and demonstration of *B. botulinus*.

METHODS OF ANAEROBIOSIS

Most of the descriptions of *B. botulinus* in the literature designate the organism as a strict anaerobe. It is stated that this bacterium can grow only in the presence of atmospheric oxygen, provided symbiotic organisms, as for example *sarcina*, cocci or *B. subtilis*, are present (van Ermengem,¹⁸ Shippen¹⁹ and others).

Studies conducted by F. Field and C. C. Dozier and numerous incidental observations made in this laboratory indicate that *B. botulinus* can probably grow in an environment which contains about $\frac{2}{3}$ atmospheric oxygen. Loosely packed, damp hay or soil inoculated with detoxified spores will become toxic in the presence of air. The aerobic index determined for *B. botulinus* according to the procedure of Harris in a 2% glucose van Ermengem broth varies from 60/0 to 110/0. Pure and mixed cultures of *B. botulinus* grow abundantly in beef heart digest broth which is not stratified with petrolatum or freed from air by heating before inoculation. Toxins varying in potency from 10,000 M L D per c c for 250 gm. guinea-pigs inside of 96 hours following subcutaneous injection are regularly produced. It is frequently noted that the growth in beef heart cultures is more rapid and vigorous when the culture flasks are partially exhausted. The following explanation can

¹⁸ Reference 12, p. 922.

¹⁹ Arch. Int. Med., 1919, 23, p. 388.

be offered for this observation. The soil specimens are suspended in bottles with an equal part of salt solution; after heating for from 1 to 2 hours, the content is thoroughly mixed with freshly boiled, oil-stratified beef heart digest medium. In the course of shaking, the medium retains a considerable amount of air, which escapes slowly from the culture fluid. In this connection, it should be stated that the paraffin oil stratified on the medium is used to prevent undue evaporation of the fluid during the prolonged incubation, which is necessary to test the sterility of the broth. This oil usually forms an emulsion with the soil-beef heart material when shaken and increases the air content of the culture. For this reason, it has been found advantageous to mount a rubber stopper fitted with a bent glass tubing in the neck of the bottle and to exhaust the content with an electric pump. In order to procure a vacuum of from 5 to 15 mm., it is necessary to cover the stoppers and the bottle neck with "Imperial cement." The glass tubing leading to the pump is allowed to seal itself off by placing it in the middle of a small Bunsen flame. In order to possess comparative data, both the experiments with van Ermengem and beef heart digest medium must be carried out by this method. As already stated, the violent gas production in the glucose medium leads frequently to breakage of the bottles, and it has been found necessary to loosen the stoppers on the third or fourth day of incubation.

Since the beef heart digest medium has been used in a routine way for the enrichment of *B. botulinus*, attempts have repeatedly been made to replace the procedure of exhaustion by stratifying the medium with petrolatum. As a rule, exceedingly satisfactory results have been obtained, but it has been found necessary to add the melted petrolatum after the heated samples have been mixed with the medium. Some observations indicate that this method is not entirely free from dangers of contamination, and it has therefore been used only in exceptional instances. It must, however, be emphasized that the methods mentioned are needed only when mass cultures, using from 20 to 100 gm. of material, are used. Under ordinary circumstances, perfect enrichment and toxin production is obtained in test tubes containing freshly boiled beef heart digest mediums without petrolatum.

PRECAUTIONS AGAINST LABORATORY CONTAMINATIONS

The relatively frequent demonstration of *B. botulinus* in specimens of soil, vegetables, etc., was so startling and contrary to previous experiences that the question of contaminated culture mediums, glassware, etc., was seriously considered and accordingly investigated. In view of the precautions that have been taken to eliminate any possibility of cross contamination, this source of error can be absolutely excluded.

As early as 1908, T. Smith²⁰ called attention to the inefficiency of discontinuous steaming of culture mediums. He found that in the routine preparation of diphtheria toxin *B. tetani* survived the repeated steamings. Anybody working with anaerobes frequently makes the observation that some tubes of the pasty culture mediums, as for example brain or cooked beef heart mediums, may contain *B. sporogenes* after prolonged incubation. For technical reasons, it has been

²⁰ Jour. Am. Med. Assn., 1908, 50, p. 929.

customary to sterilize these mediums by intermittent heating in the Arnold sterilizer. This procedure may be adequate for a laboratory in which little or no anaerobic spore material is prepared or investigated; it becomes, however, an unreliable method when hundreds of mass cultures, some containing billions of most resistant spores, are being handled in the course of a single day. If one, furthermore, appreciates that the spores of *B. botulinus* can survive 4 to 5 hours' boiling, and that the heated spores may frequently not germinate in from one week to several months, the potential danger of inadequate sterilization is quite evident. It is a common experience that lack of proper recognition of these dangers not infrequently leads to an extensive pollution of all the equipment of a cleaning kitchen and a culture medium department, and without proper sterilization a certain percentage of preparations will be found contaminated with the organism which is being studies in the laboratory. In the examination of specimens of soil and its products, additional danger exists in the dust produced in handling such material. In this connection it appears advisable to record one of the many experiments which have been made in this laboratory to determine the distribution of the spores of *B. botulinus* on the working benches, etc.

Exper. 4 (Series 146).—The dust, etc., resting on various working benches, shelves, etc., was removed by means of sterile cotton pellets held by a sterile forceps. The cotton was placed in sterile salt and heated for 2 hours at 70 C., then mixed with 100 c.c. of sterile beef heart digest medium and treated according to the adopted procedure. The flasks were incubated for 10 days at 35 C. The results are presented in table 2.

It is evident from the data presented in table 2 that even in a laboratory in which the danger of dissemination of heat resistant spores has been fully recognized, some places are found to be contaminated. However, this and subsequent tests furnish definite assurance that the technic on the working benches on which field samples are handled is sufficiently rigid to prevent the contamination of field specimen cultures.

In order to assist workers who are confronted with a similar problem, the various methods employed are briefly summarized:

New and used glassware, discarded cultures in bottles or tubes, etc., are sterilized for 3 hours at 18 pounds' pressure. The sterilized glassware is cleaned by boiling in strong soda solution, dipped in dilute hydrochloric acid and then rinsed thoroughly in tap water. Dried and plugged test tubes, flasks, etc., are sterilized in the hot air sterilizer for 3 hours at 170 to 180 C. Culture mediums, salt solutions in sterilized containers, pipets, rubber stoppers, etc., are sterilized at 18 pounds' pressure for one hour. All mediums are

incubated for from 5 to 14 days and then resterilized for 1 hour. Since the adoption of these precautions, nonsterile mediums have not been encountered. The working benches are covered daily with fresh, sterile wrapping paper which is soaked with a 10 to 15% dilution of compound solution of cresol. The same antiseptic is used for the washing down of shelves, tables, balances, etc. Pipets, mortars, etc., infected with cultures or soil are placed either in 50% dilution of compound solution of cresol or 20% formaldehyde solution. Tests conducted by C. C. Dozier have shown that the usual antiseptic solutions employed by bacteriologists for the destruction of pathogenic nonsporulating bacteria fail to destroy the spores of *B. botulinus* in less than 8 days. Weighing and bottling of soil or other field specimens is done in a large enamel tray (50 x 50 cm.) the bottom of which is covered by a layer of 25% dilution of compound solution of cresol. For special work, a sterile inoculation room is frequently used, but in the experience of this laboratory, the sterility of culture mediums and glassware is of greater importance than the precautions against air contaminations.

TABLE 2
RESULTS OF EXPERIMENT 4

Sample	Specimen Cultures	Result of Culture
1	Dust collected from table and bench on which soil samples are weighed and distributed in flasks	Nontoxic
2	Dust from shelves of medium cabinet.....	Nontoxic
3	Dust from shelves on which sterile glassware is stored.....	Nontoxic
4	Working bench of A; at this place numerous samples of spore suspensions are counted daily	2 c c toxic for guinea-pigs in 4 days
5	Working bench of B; at this place mass cultures are prepared for serologic tests	2 c c toxic for guinea-pigs in less than 16 hours
6	Working bench of C; at this place mass cultures have been purified; hundreds of flasks have been inoculated with pure spore cultures of <i>B. botulinus</i>	Nontoxic
7	Working table used for necropsies, etc.	2 c c produced symptoms, but guinea-pig recovered
8	Wrapping paper used as covers on table and cabinet mentioned under (1) and (2)	Nontoxic
9	Dust from the floor of an incubator room.....	2 c c toxic for guinea-pig in less than 16 hours
10	Dust from shelves and tables of cleaning kitchen used for the storage of culture flasks, etc., previous to sterilization; 2 samples each	2 c c toxic for guinea-pig in less than 16 hours

AMOUNT AND PREPARATION OF FIELD MATERIAL TO BE EXAMINED

Spoiled and toxic vegetable foods contain, as a rule, numerous viable spores of *B. botulinus*; enrichment methods are then unnecessary, and the organisms can frequently be isolated in deep agar shake cultures. Preliminary studies with soil specimens which as a result of enrichment cultures were known to contain spores of *B. botulinus*, failed to furnish the desired information relative to the number present in a weighed amount of earth. Numerous samples were cultivated by distributing from 1 to 10 gm. of heated soil by the dilution method either in deep agar shake tubes or in blood-glucose-agar plates, but toxin-producing colonies could not be isolated. It was therefore necessary to estimate the relative numerical distribution of the spores of *B. botulinus* by indirect methods. Theoretically, it was assumed that within certain limits the larger a sample examined the greater the probability of procuring toxic enrichment cultures. Furthermore, the use of specimens varying in amounts from 10 to 50 gm. partially eliminated as a factor of importance the well known uneven distribution of the spores in soil. These considerations were,

however, of little value without a definite knowledge relative to the sensitiveness of the enrichment method. The question—"How many viable spores must be present in a soil sample to produce a toxic enrichment culture?"—was therefore submitted to an experimental study.

Heating of field specimens in order to eliminate nonsporulating aerobes and undesirable anaerobes is commonly used by the anaerobist, but deserves additional investigation so far as *B. botulinus* is concerned. Dickson and Burke⁷ heated the contents of crops and gizzards or of the intestinal canal of poisoned chickens for one hour at 60 C. In a more recent publication, however, Burke⁸ failed to state the method of preparation of the specimens she tested for the presence of *B. botulinus* spores. The impression is conveyed that the various samples of vegetables, hay, manure and soil were not subjected to heat.

The experiments dealing with the various phases considered in the foregoing are herewith detailed. Numerous quantitative tests using van Ermengem's medium and beef heart peptic digest liver broth have been carried out. Samples of soil or fruits were cultivated in amounts of 1, 10, 20 and 50 gm. Two experiments are presented to illustrate the character of the tests.

TABLE 3
RESULTS IN EXPER. 5

Specimen	Amount of Chopped Olives, Gm.	Culture Media	
		van Ermengem	Beef Heart Peptic Digest Broth
Unbroken olives.....	10.0	Nontoxic	Toxic; type B isolated
From tree.....	1.0	Nontoxic
Bird pecked olives.....	10.0	Toxic; weak toxin	Toxic; type B isolated
	1.0	Toxic; weak toxin
Olives buried in soil.....	10.0	Nontoxic	Toxic; weak toxin
	1.0	Nontoxic

Exper. 5 (Ser. 4).—Three lots of olives collected from a ranch in Northern California were chopped up separately, and heated for one hour at 65 C. Ten and 1 gm. lots were cultivated in 100 cc amounts of van Ermengem's and beef heart digest broth. The cultures were incubated at 38 C. for 30 days. The results are shown in table 3.

The superior properties of the beef heart broth as an enrichment medium are well illustrated in this experiment. Specimens which give toxic cultures are either nontoxic in van Ermengem's broth, or the toxin is not sufficiently potent for proper identification. The number of viable spores after heating for one hour at 65 C. is small in the unbroken and bird pecked olives, inasmuch as 1 gm. samples furnish only weakly toxic or nontoxic cultures.

Exper. 6 (Ser. 122 B).—Virgin soil collected from a recent rockslide in Yosemite Valley was distributed in 1, 10, 20 and 50 gm. lots in bottles con-

taining 10 cc of salt solution. The suspensions were heated for 2 hours at 70 C., then mixed with 100 cc of beef heart medium and incubated for 10 days at 35 C. The 1 gm. cultures were nontoxic, while the 10, 20 and 50 gm. series produced toxins which were fatal in 2 cc amounts to guinea-pigs in less than 16 hours. *B. botulinus*, type A, was isolated from several cultures.

From these and similar experiments, it appears advisable to recommend the cultivation of fairly large vegetable and soil specimens, unless the material is relatively rich in other saccharolytic and proteolytic anaerobes. This reservation is prompted by the observation that, whereas small quantities (10 gm.) of manured soil furnish toxic cultures, 50 or 100 gm. specimens reveal no demonstrable toxin when treated under identical conditions. An explanation for these differences will be attempted later in the discussion on the deterioration of the toxin in anaerobic cultures.

TABLE 4
RESULTS IN EXPER. 7

Number of Bottle	Soil	Number of Spores and Rods Inoculated	Result
1	10 gm. (39. II-V)	6 spores and 3 rods	2 cc of culture toxic for guinea-pig in 24 hours
2	10 gm. (39. II-V)	13 spores and 3 rods	2 cc of culture toxic for guinea-pigs in 24 hours
3	10 gm. (39. II-V)	5 spores and 3 rods	Nontoxic
(4)	10 gm. (39. II-V)	10 spores and no rods	2 cc produced symptoms and death in 17 days

A series of tests has been carried out in order to determine the approximate number of spores which must be present in specimens of soil, hay, etc., in order to produce a toxic enrichment culture.

The question: "How many spores inoculated into culture mediums containing heated soil will germinate and produce toxin in the presence of other bacteria?" was approached with the aid of Barber's single cell isolation method. Two types of experiments can be reported.

Exper. 7.—Five bottles containing 100 cc of beef heart peptic digest broth and 10 gm. of heated mixed soil (2 hours at 70 degrees), which on repeated tests was found free from *B. botulinus*, were inoculated with a few unheated spores and vegetative rods of *B. botulinus*, strain 38, picked by the Barber method from a 10-day culture in plain gelatine. The cultures were kept at 35 C. for 10 days. The results are summarized in table 4.

Exper. 8.—The experiment was similar to that reported under *Exper. 7*; however, the soil and the spores (strain 62) picked under the microscope were heated simultaneously for 2 hours at 70 C. The suspension was then mixed with the beef heart digest broth, exhausted and incubated for 10 days at

37 C. The spores of *B. botulinus* were subjected to the same treatment as was customary in the testing of field specimens. The results are shown in table 5.

Unheated spores added to heated soil emulsions in beef heart peptic digest broth are capable of germination and multiplication. It is, how-

TABLE 5
RESULTS IN EXPER. 8

Number of Bottle	Soil	Number of Spores	Result
1	5 gm. of (84 c. VIII)	2	2 c c produced symptoms and death on the 16th day
(2)	5 gm. of (84 c. VIII)	3	Nontoxic
(3)	5 gm. of (84 c. VIII)	4	Nontoxic
(4)	5 gm. of (84 c. VIII)	7	Nontoxic
(5)	5 gm. of (84 c. VIII)	9	Nontoxic

TABLE 6
RESULTS IN EXPER. 9

Series	20 Gm. Soil	Medium	Number of Spores Added Previous to Heating	Result After Incubation for 10 Days at 37 C.	Result After Incubation for 10 Days at 28 C.
A	1 Nonsterile	Beef heart digest	1,000 to 10,000	2 c c toxic in 16 hours	Nontoxic
	2 Sterile	Beef heart digest	1,000 to 10,000	2 c c toxic in 16 hours	2 c c toxic in 16 hours
	3 Sterile	Peptic digest no meat	1,000 to 10,000	2 c c toxic in 16 hours	2 c c toxic in 16 hours
B	4 Nonsterile	Beef heart digest	100 to 1,000	2 c c toxic in 48 hours	Nontoxic
	5 Sterile	Beef heart digest	100 to 1,000	2 c c toxic in 16 hours	2 c c toxic in 16 hours
	6 Sterile	Peptic digest	100 to 1,000	2 c c toxic in 16 hours	2 c c toxic in 16 hours
C	7 Nonsterile	Beef heart digest	10 to 100	2 c c toxic in 5 days	Nontoxic
	8 Sterile	Beef heart digest	10 to 100	Toxic in 24 hours	Toxic in 16 hours
	9 Sterile	Peptic digest	10 to 100	Toxic in 16 hours	Toxic in 16 hours
D	10 Nonsterile	Beef digest	1 to 10	Nontoxic	Nontoxic
	11 Sterile	Beef digest	1 to 10	Toxic in 16 hours	Nontoxic
	12 Sterile	Peptic digest	1 to 10	Toxic in 16 hours	Nontoxic

ever, impossible to obtain identical results by repeating the same procedure; only 2 or 3 of the 10 flasks inoculated with the same number of spores and rods may give toxic cultures. Moreover, these experiments are not a duplicate of the method employed for the enrichment of

field specimens and therefore are of little value to test the sensitiveness of the culture medium, etc. Nevertheless, they indicate definitely that a few viable spores accidentally introduced as contaminations may produce toxic cultures and furnish misleading results. In the light of the

TABLE 7
RESULTS IN EXPER. 11

Series	Soil, Gm.	Medium	Number of Spores Added Previous to Heating	Result After 10 Days' Incubation Subcutaneous Injection
A	1 50	Beef heart digest broth	10,000 to 100,000	1 c c toxic in 24 hours
	2 50	Beef heart digest broth	10,000 to 100,000	1 c c toxic in 30 hours
	3 50	van Ermengem mod.	10,000 to 100,000	1 c c toxic in 48 hours
	4 50	van Ermengem mod.	10,000 to 100,000	1 c c toxic in 30 hours
B	5 50	Beef heart digest broth	1,000 to 10,000	1 c c toxic in less than 16 hours recovered organism
	6 50	Beef heart digest broth	1,000 to 10,000	1 c c toxic in 40 hours
	7 50	van Ermengem mod.	1,000 to 10,000	1 c c toxic in 48 hours
	8 50	van Ermengem mod.	1,000 to 10,000	1 c c toxic in 30 hours
C	9 50	Beef heart digest broth	100 to 1,000	Nontoxic
	10 50	Beef heart digest broth	100 to 1,000	2 c c toxic in 60 hours
	11 50	van Ermengem mod.	100 to 1,000	Nontoxic
	12 50	van Ermengem mod.	100 to 1,000	Nontoxic
D	13 50	Beef heart digest broth	10 to 100	Symptoms, died 18th day
	14 50	Beef heart digest broth	10 to 100	2 c c toxic in 48 hours
	15 50	van Ermengem mod.	10 to 100	Nontoxic
	16 50	van Ermengem mod.	10 to 100	Nontoxic
E	17 50	Beef heart digest broth	No spores	2 c c nontoxic
	18 50	Beef heart digest broth	No spores	2 c c nontoxic
	19 50	van Ermengem mod.	No spores	2 c c nontoxic
	20 50	van Ermengem mod.	No spores	2 c c nontoxic
Controls	4 tubes each	Beef heart digest broth	10,000 to 100,000	All toxic, heated and unheated
	4 tubes each	Beef heart digest broth	1,000 to 10,000	All toxic, heated and unheated
	4 tubes each	Beef heart digest broth	100 to 1,000	All toxic, heated and unheated
	4 tubes each	Beef heart digest broth	10 to 100	Heated; 2 toxic, 2 nontoxic; unheated; 3 toxic, 1 nontoxic

observations made in exper. 7, the elaborate precautions against contaminations appear fully justified.

A few spores added to soil suspensions and subsequently heated in the customary manner give nontoxic or weakly toxic cultures. Fre-

quent repetitions of exper. 8 with as many as 40 spores fail to produce toxic cultures. These observations are confirmed by the following experiments.

Exper. 9.—Twenty gm. of soil repeatedly found free from spores of *B. botulinus* were suspended in salt solution. A mixture of spores produced in gelatine broth (strains 58 and 62) were counted in a Helber chamber; dilutions of the spore suspension were made in salt solution and 1 cc each of the various dilutions were added to the nonsterile and sterile soil. The suspensions were heated for one hour at 70 C., mixed with beef heart digest broth and exhausted. One series of flasks was incubated at 35 C., while the other was kept at 28 C. for 10 days. The results are shown in table 6.

Exper. 10.—The same experiment was repeated with the same soil specimens and with spores of strain 90. The same results were obtained as in exper. 9.

Exper. 11.—A mixture of 8 different soil specimens, free from spores of *B. botulinus* was distributed in 50 gm. amounts in a series of bottles and then suspended in salt solution with a P_H of 7.4. Four bottles received 1 cc each of varying dilutions of an unheated spore suspension of *B. botulinus*

TABLE 8
RESULTS IN EXPER. 12

Series	20 Gm. Soil	Medium	Number of Spores Added Previous to Heating	Result After 10 Days' Incubation
(1)	Nonsterile	Beef heart peptic digest	400 to 600	Toxic in less than 16 hours
(2)	Sterile	Beef heart peptic digest	400 to 600	Toxic in less than 16 hours
(3)	Nonsterile	Beef heart peptic digest	40 to 60	Nontoxic
(4)	Sterile	Beef heart peptic digest	40 to 60	2 cc produced symptoms but guinea-pig recovered

strain 97 (140 hours old). The dilutions were prepared with great care and were controlled by deep agar shake cultures. The soil-spore suspensions were heated for one hour at 70 C., mixed with either beef heart digest or van Ermengem medium and incubated for 10 days at 35 C. The results are presented in table 7.

Exper. 12.—The number of spores added to the soil suspensions was in this experiment determined by the plating of heated and unheated spore dilutions in blood agar. Spores (strains 90 and 47) which were dried for 24 hours on sand over sulphuric acid were used. The treatment of the inoculated soil suspensions was similar to that employed in the preceding experiments. The results are shown in table 8.

The data presented in exper. 9, 10, 11 and 12 conclusively indicate that toxic soil enrichment cultures are regularly obtained, provided the specimens contain at least from 100 to 1,000 viable heat resistant spores. Exceptions may occur, as, for example, in exper. 11, ser. C and D, one sample containing less than 100 spores proved sufficiently toxic to kill

a guinea-pig in 48 hours, while of those containing from 100 to 1,000 spores, only one furnished a toxic enrichment culture. It is obvious that in order to demonstrate the existence of *B. botulinus* spores in soil specimens, adequate samples of not less than 10 gm. should be chosen. When the question is sufficiently important, it is recommended to prepare a series of cultures containing 1, 10, 20, 50 and 100 gm. of soil.

The heating of soil, vegetable or food specimens previous to making of cultures is a recognized practice among workers with sporulating anaerobes. The methods which have proved satisfactory in this laboratory have recently been reported by H. H. Heller.²¹ Concerning *B. botulinus*, nothing definite was known when these studies were begun, but by analogy it was assumed that toxic enrichment cultures could probably be procured more readily after destroying the nonsporulating bacteria. Rabinowitsch,²² for example, reports that the heating of specimens used for the isolation of *B. tetani* is very important. Thus, toxic cultures only were procured when the 2% glucose broth in which 1 lb. of strawberries had been washed was heated for 30 minutes at 80 C. In judging the advantages and disadvantages of heated specimens for enrichment cultures, it appears advisable to consider the heat resistance of the spores of *B. botulinus*. In the American literature the conception prevails that the spores produced in various culture mediums are very resistant to heat, while in Europe the original observations of van Ermengem, who found the spores to be readily killed after an exposure to 80 C. for 30 minutes, are assumed to be correct. It must be remembered, however, that the germination of the spores may be considerably retarded when exposed to heat and that the resistance of the prevailing spores, as found in nature, is unknown. In view of these mentioned conditions, it is suspected that frequently nontoxic enrichment cultures, and consequently the studies dealing with the distribution of the spores of *B. botulinus*, furnish an erroneous conception of actual conditions. Experiments to be reported indicate that it is technically impossible to prove this point. Thus, unheated soil specimens, containing viable spores of *B. botulinus* in small numbers, furnish nontoxic, or weakly toxic, enrichment cultures. The various concomitant aerobes or anaerobes apparently overgrow *B. botulinus*, or the enzymes of these bacteria destroy the generated toxin.

²¹ Jour. Bacteriol., 1921, 6, p. 445.

²² Arch. f. Hyg., 1907, 61, p. 103.

From a practical standpoint, it appears important to know the special distribution of those spores which are very resistant to heat. It is this type which, at least in this country, causes the majority of botulism outbreaks. A number of experiments have been carried out to investigate this question. Two are described in detail to illustrate the methods and results.

Exper. 13.—A mixture of 6 different soil specimens previously found to be free from spores of *B. botulinus* was distributed in 20 gm. amounts into a series of bottles and suspended in salt solution. Two bottles received 1 cc

TABLE 9
RESULTS IN EXPER. 13

Series	Treatment Previous to Making of Cultures	Number of Spores Added	Result After 10 Days' Incubation at 37 C.
A	1 Not heated	1,000 to 10,000 in soil	2 cc produced symptoms but guinea-pig recovered
	2 Heated at 70 C.	1,000 to 10,000 in soil	2 cc toxic in 18 hours
	3 Heated at 70 C.	1,000 to 10,000 in salt solution	2 cc toxic in 18 hours
B	1 Not heated	100 to 1,000 in soil	2 cc toxic in 5 days
	2 Heated at 70 C.	100 to 1,000 in soil	2 cc toxic in 5 days
	3 Heated at 70 C.	100 to 1,000 in salt solution	2 cc toxic in 18 hours
C	1 Not heated	10 to 100 in soil	Nontoxic
	2 Heated at 70 C.	10 to 100 in soil	2 cc toxic in 16 days
	3 Heated at 70 C.	10 to 100 in salt solution	2 cc toxic in 18 hours
D	1 Not heated	1 to 10 in soil	2 cc nontoxic
	2 Heated	1 to 10 in soil	2 cc nontoxic
	3 Heated	1 to 10 in salt solution	2 cc toxic in 48 hours
E	1 Not heated	No spores	Nontoxic
	2 Heated	No spores	Nontoxic

each of varying dilutions of an unheated spore suspension of *B. botulinus* 97. As controls, bottles containing salt solution were inoculated with the same suspension; one soil-spore and the control salt solution spore suspension culture were heated for one hour at 70 C. The unheated and heated bottles were filled with beef heart digest medium, exhausted and incubated for 10 days at 35 C. The results are shown in table 9.

Exper. 14.—The intestinal washings of one human case of botulism were distributed into 10 bottles each containing 75 cc. A formed stool specimen was carefully emulsified and also divided into 6 portions of 50 cc each. One-half of the bottles of each series of stool specimens was heated for 1½ hours at 60 C., mixed with beef heart digest broth, exhausted and incubated at 37 C. for 10 days. The results of this experiment are shown in table 10.

From the results obtained in expts. 13 and 14, one may conclude that soil and stool specimens must be heated previously in order to obtain toxic enrichment cultures. The outcome of these tests is so conclusive that further comment is unnecessary and heating of such specimens for 2 hours at a temperature of from 60 to 70 C., or one hour at 80 C., must be made a routine practice.

It should be stated in this connection that very acid food samples, etc., should be heated only after complete neutralization. The procedure suitable for this purpose will be considered in subsequent paragraphs of this paper.

TABLE 10
RESULTS IN EXPER. 14

Specimen	Treatment Previous to Making of Cultures	Result After 10 Days' Incubation at 37 C.
Intestinal washing 1.....	Heated	2 c c toxic in 24 hours) Isolated B. botu-
2.....	Heated	2 c c toxic in 18 hours) linus type A
3, 4, 5, 6.....	Heated	2 c c nontoxic
Intestinal washing 7, 8, 9, 10, 11, 12	Not heated	All nontoxic
Formed stool 1.....	Heated	2 c c toxic in 18 hours, B. botulinus
2, 3.....	Heated	type A
Formed stool 4, 5, 6.....	Not heated	Nontoxic
		All nontoxic

PERIOD OF INCUBATION

Two questions deserve experimental investigation, namely: 1. What is the optimum temperature for the growth of *B. botulinus* in mixed cultures? 2. What period of incubation at a temperature of 28 to 37 C. furnishes toxins of the highest potency?

It is well known that van Ermengem,¹⁴ Römer,²³ Würcker²⁴ and other European bacteriologists described strains of *B. botulinus* which developed quickly and abundantly at a temperature of from 20 to 30 C. Landman²⁵ and later Leuchs²⁶ noted, however, that one strain of *B. botulinus* isolated from string beans (Darmstadt outbreak) grew very well at 37 C., although the toxin production was better at 24 C. In a recent compilation, Bitter⁴ concludes that all the strains of *B. botulinus* known in Germany until 1919 may grow in artificial culture mediums at a temperature of 37 C., but that the microbe produces the most potent toxin when incubated at from 25 to 30 C. Up to three or four years ago these views have guided the workers in the United States, and the early studies made on *B. botulinus* were conducted with cultures which were kept at 28 C. The publications of Nevin,⁶ Shippen,¹⁹ Thom, Edmonson and Giltner,⁹ Orr²⁷ and of Burke,¹ which report typical

²³ Centralbl. f. Bakteriöl., Orig. 1900, 27, p. 857.

²⁴ Sitzungsber. d. physikal-medizin. Sozietät in Erlangen, 1909, 41, p. 209.

²⁵ Hyg. Rundschau, 1904, 14, p. 449.

²⁶ Ztschr. f. Hyg. u. Infektionskrankh., 1910, 65, p. 55.

²⁷ Jour. Infect. Dis., 1922, 30, p. 118.

growth and excellent toxin production at 37 C. with American strains of *B. botulinus* isolated from a variety of food and soil products, were available when the studies presented in this paper were undertaken. As far as enrichment cultures with soil, hay and vegetable specimens is concerned, it should be stated that Burke⁸ adopted an incubation period of from 2 to 6 months at 28 C. This worker, however, states that "this incubation period was too long, as the average life of *B. botulinus* in broth cultures is about three months." A higher percentage of positive cultures could have been obtained, in the opinion of Burke, provided the cultures had been incubated for only 6 to 10 weeks at 28 C. Quite recently, Kendall²⁸ recommended that *B. botulinus* should be grown at 30 instead of at 38 C.

It is evident from this review that the information regarding the optimum temperature conditions for the growth of *B. botulinus* in enrichment cultures is incomplete and contradictory. The knowledge relative to the incubation period required for the production of a toxin of highest potency is equally unsatisfactory. For example, Thom, Edmonson and Giltner⁹ usually obtained a toxin with a potency of 0.001 c c for guinea-pigs in cultures incubated for 6 days at 37 C.; while the best toxin (M L D 0.0001 c c) was produced at 35 C. for 28 days. Orr²⁹ tested toxins which had been generated in cultures kept for 3 weeks at 37 C.; the M L D for mice did not exceed 0.0001 c c. Systematic studies to determine the optimum incubation period for *B. botulinus* in a suitable culture medium have not been carried out. In fact, the first hundred enrichment cultures prepared in this laboratory were incubated empirically for 4 weeks at 37 C. It was, however, noted that specimens which were originally toxic, containing *B. botulinus* in large numbers, would produce weakly toxic enrichment cultures. Theoretically such cultures could only result from (1) deterioration of the toxin at body temperature, or (2) destruction of the toxin molecule by the concomitant bacteria present in the enrichment culture. A systematic study of this question was therefore undertaken by a number of workers in this laboratory. The findings which have a direct bearing on the problem under consideration are briefly mentioned:

A comparative study of over 100 strains of *B. botulinus* from various sources has shown that growth and toxin production is best at a temperature of 35 C. According to thermographic records, the incubation rooms installed in this laboratory have an average temperature of 35 C. Pure cultures of *B. botulinus* in beef heart digest medium produce on the 10th to 12th day of incubation at 35 C. toxins of the maximum potency; the M L D (96 hours) for a guinea-pig of 250 to

²⁸ Ibid., 1921, 29, p. 231.

²⁹ Jour. Med. Res., 1921, 42, p. 127.

300 gm. is usually from 0.00002 c c to 0.0001 c c. Incubation at the same temperature for 15, 20, 25, 30, etc., days leads to a distinct deterioration of the toxin. These studies will be reported in a series of papers, dealing with the biochemical activities of *B. botulinus* in various mediums. The behavior of the toxin in enrichment cultures is similar to that in pure cultures; as a rule, the deterioration is more rapid, as will be noted in exper. 15 and 16.

Exper. 15.—A series of enrichment cultures which proved very toxic on the 10th day were kept in the incubator for 30 days. Additional toxin tests were made on the 20th and 30th day, and the data are presented in table 11.

TABLE 11
RESULTS IN EXPER. 15

Number of Sample	Nature of Specimens	1st Test 10th Day at 35 C.	2d Test 20th Day at 35 C.	3d Test 30th Day at 35 C.	Type of <i>B. botu- linus</i>
21 c. M.	Soil	2 c c toxic in 16 hours	2 c c toxic in 24 hours	2 c c toxic in 36 hours	A
40 M. M.	Soil	2 c c toxic in 24 hours	2 c c toxic in 6 days	2 c c nontoxic	A
202 V	Soil	2 c c toxic in 16 hours	2 c c toxic in 48 hours	2 c c toxic in 12 days	A
203 XIII	Beanstalk	2 c c toxic in 16 hours	2 c c toxic in 48 hours	2 c c toxic in 5 days	B
205 XIV	Soil	2 c c toxic in 16 hours	2 c c toxic in 5 days	2 c c nontoxic	A
207 XII	Soil	2 c c toxic in 16 hours	2 c c toxic in 16 hours	2 c c toxic in 24 hours	B
207 XII	Soil	2 c c toxic in 16 hours	2 c c toxic in 3 days	2 c c toxic in 6 days	B
209 XXIII	Soil	2 c c toxic in 48 hours	2 c c toxic in 10 days	2 c c nontoxic	A
209 XX	Cucumber	2 c c toxic in 16 hours	2 c c toxic in 24 hours	2 c c toxic in 3 days	A
214 40	Soil, England	2 c c toxic in 40 hours	2 c c toxic in 5 days	2 c c nontoxic	B
214 60	Soil, England	2 c c toxic in 16 hours	2 c c toxic in 24 hours	2 c c toxic in 24 hours	B
217 III	Soil	2 c c toxic in 16 hours	2 c c toxic in 4 days	2 c c toxic in 15 days	A

Exper. 16.—The filtered toxin of strain 38 ($M L D \pm 0.0001$) was divided into two large test tubes, 75 c c each. To tube 1, were added 5 c c of the sediment of a 10 days' soil culture which was nontoxic and free from *B. botulinus*, but contained various aerobes and anaerobes; and to tube 2, 5 c c of a carefully sterilized soil suspension were added. Both tubes were incubated at 35 C., and the toxin content determined on the 5th and 15th days. The results are shown in table 12.

The findings in exper. 15 and 16 indicate that the toxin of *B. botulinus* deteriorates when kept at 35 C. The rate of destruction is not influenced entirely by the concomitant bacteria of the enrichment culture, but probably depends on the degree of toxicity reached on the 10th day. In other words, a highly toxic enrichment culture kept near

body temperature may remain fatal for guinea-pigs, while weakly toxic cultures become nontoxic during the same period of incubation at the same temperature. It naturally suggested itself to study the influence on toxicity of incubation at 28 C. A number of tests similar to those reported under exper. 10 were carried out. Without going into details, it was noted that frequently nontoxic cultures were obtained at 28 C., even after prolonged observation, although incubation at 35 C. produced satisfactory specimens. Lack of proper incubation room facilities made it unpractical, however, to handle many hundreds of cultures at a temperature of 28 C., although the observations were sufficiently numerous to warrant a positive deduction.

TABLE 12
RESULTS IN EXPER. 16

Tube	M L D at Beginning	5th Day at 35 C.	15th Day at 35 C.
Toxin + soil culture.....	0.0001 c c	0.01 c c (growth)	0.6 c c
Toxin + sterile soil.....	0.0001 c c	0.01 c c (sterile)	0.6 c c

TABLE 13
RESULTS IN EXPER. 17

Specimens	Time Required for 2 c c of the Centrifugalized Supernatant Fluid of a Culture to Kill Guinea-Pigs of 300-350 Gm.			
	24 Hours	48 Hours	5 Days	10 Days
1 gm. of soil	Nontoxic	Nontoxic	Nontoxic	Nontoxic
10 gm. of soil	Nontoxic	Nontoxic	Symptoms	4 days = 1 M L D
20 gm. of soil	Nontoxic	48 hours	24 hours	16 hours
50 gm. of soil	Symptoms	48 hours	24 hours	16 hours

In order to prove that the most potent toxin is produced on the 10th day of incubation at 35 C., cultures were made from a number of virgin soil samples, which were known to contain spores of *B. botulinus*. Toxin determinations were made at varying times during the incubation. One of these experiments is herewith detailed.

Exper. 17.—Virgin soil from Yosemite Valley was distributed in bottles suspended in salt solution and heated for 2 hours at 70 C. The suspensions were mixed with peptic digest broth, exhausted and incubated at 35 C. The presence of toxins was determined by subcutaneous inoculations of guinea-pigs. The results are shown in table 13.

The evidence justifies the conclusion that an incubation period of 10 days at 35 to 37 C. in beef heart peptic digest broth furnishes the optimum conditions for securing highly toxic enrichment cultures.

Observations to be recorded in subsequent paragraphs also indicate that such cultures are well suited for the isolation and purification of *B. botulinus* from soil specimens.

STORAGE OF CULTURES

The examination of a large series of cultures, limited assistance, continuous observation of the inoculated guinea-pigs and occasionally a shortage of these animals necessitated a prolonged storage of the enrichment cultures. As each toxic culture was repeatedly tested and typed with antitoxins, it was essential to possess a practical method which would preserve the toxicity of the cultures at its original titer. The facts presented in the preceding paragraphs and the observations made on numerous cultures suggested storage at a low temperature. It was repeatedly noted that bottles containing a strong toxin would become nontoxic or weakly toxic and unsuitable for identification tests when kept during the summer months in a warm corner of the laboratory (about 30 C.). Facilities to store hundreds of bottles in the refrigerator were not available, but storage in dark cabinets at an average room temperature of 18 to 22 C. was practical. The value of this procedure has been tested and one of the experiments is herewith detailed.

Exper. 18.—Several enrichment cultures were stored under different conditions and the potency of the toxins estimated on guinea-pigs by the usual procedures. The results can be presented as follows:

Specimen Group 1: Originally 2 cc toxic for 250 gm. guinea-pig in 48 hours; at 18 to 22 C. for 20 days killed in 16 hours.

Specimen Group 2: Originally 2 cc toxic for 250 gm. guinea-pig in 48 hours; at 2 to 5 C. for 20 days killed in 40 hours.

Specimen Group 3: Originally 2 cc toxic for 250 gm. guinea-pig in 48 hours; at 35 C. for 20 days produced only symptoms, and the guinea-pig recovered.

Based on this and similar observations, it was concluded that the toxicity of an enrichment culture does not decrease; in fact it may increase when kept at a temperature of 18 to 22 C. Storage at this temperature has therefore been adopted as a routine procedure.

IDENTIFICATION OF THE TOXIN

In order to segregate the toxic from the nontoxic cultures, the following method was chosen: Ten cc of the supernatant fluid of the culture were removed, care being exercised to avoid stirring up the meat and soil sediment. These samples were centrifugalized for one hour at high speed (3,000-4,000 revolutions). One to 2 cc of the perfectly clear culture fluid were inoculated subcutaneously on the median abdominal line of a guinea-pig weighing not

more than 400 gm. In case the animals succumbed to an intoxication³⁰ without exhibiting the symptoms and necropsy findings of other anaerobic infections, as "malignant edema" or tetanus, the culture was considered suspicious and the nature of the poison was determined by an antitoxin neutralization test. In 90% of the tests it was possible to eliminate the nontoxic samples by this procedure. The centrifugalized cultures were only filtered when other pathogenic anaerobic bacteria, as *B. oedematiens*, *vibrio septique* produced misleading symptoms and lesions. In some instances, the centrifugalized cultures were fed instead of injected. This procedure was adopted for the following reasons: 1. Filtration of large series of cultures is exceedingly time consuming and expensive. The Mandler candles on the market are of inferior quality and necessitate frequent replacement. 2. A considerable portion of the toxin is absorbed by the filter and weakly toxic cultures become unsuitable for toxin-antitoxin tests. 3. Feeding of large series of guinea-pigs is a tedious process and exceedingly inaccurate. In the majority of instances, the animals refuse to swallow or regurgitate the foul smelling culture fluid. 4. Subcu-

TABLE 14
RESULTS OF EXPERIMENTS TO ESTIMATE APPROXIMATE STRENGTH OF TOXINS IN
ENRICHMENT CULTURES

Specimen	2 c c Killed 250 Gm. Guinea-Pig	Minimal Lethal Dose for 250 Gm. Guinea-Pigs Within 96 Hours, C c
158 soil type A.....	<16 hours	0.0002
156 stool type A.....	±28 hours	0.005
158/7 soil type A.....	+16 hours	0.0001
200 a III soil type B.....	+16 hours	0.0001
219 liver necropsy type A.....	<18 hours	0.00005
220/2 soil type B.....	48 hours	0.0005
220/10 soil type A.....	24 hours	0.01
221 A string beans type A.....	18 hours	0.00005
221 B string beans type A.....	18 hours	0.0001 (48 hours)
Exper. 11. A. L.	±30 hours	0.01
Exper. 11. A. 2.....	±24 hours	0.005

taneous injections allow quantitative tests and furnish additional information with regard to the distribution of *B. tetani* and *vibrio septique*. Intraperitoneal inoculations, on the other hand, frequently produce shock and sudden death, probably as a result of the various biogenous amines, etc., present in the cultures. Weakly toxic cultures are only detected following the injection of at least 2 c c, and therefore mice are found unsuitable and more expensive.

In order to estimate the approximate strength of the toxins present in enrichment cultures, a series of minimal lethal dose determinations was carried out. A few of the results are presented in table 14.

The results presented in table 14 indicate that soil enrichment cultures may produce exceedingly potent toxins. Cultures which are

³⁰ The necropsy findings on guinea-pigs which succumb to the toxin of *B. botulinus* are briefly as follows: A slight infiltration or edema, with or without hemorrhages, or frequently no macroscopic changes at the site of inoculation; marked congestion of the abdominal viscera with dark colored liver and spleen; in very acute intoxications these lesions are absent. Frequently a distended bladder and a rather dry appearance of the serous linings. Lungs always hyperemic and may show large or small pleural suffusions or petechiae.

fatal to guinea-pigs in 2 c.c. amounts in less than 4 days can be used for identification tests. Furthermore, several hundred observations have demonstrated that only those cultures which kill in less than 18 hours are suitable for the isolation of *B. botulinus* in a pure state. Toxins producing symptoms in 3 to 4 days leading to death on the 5th to 8th day or even later have been classified as "weak toxins." They cannot be typed, but their significance and the methods employed in preparing such specimens for identification will be treated in the next article.

The toxin identification tests were usually conducted on guinea-pigs; a limited number of cultures were also tested by intraperitoneal injections on mice. The antitoxins used were all monovalent type A and B horse or goat serums, with an antitoxin content of from 10 to 200 units per c.c.³¹ For routine tests, three animals were inoculated subcutaneously or intraperitoneally in the following manner:

TABLE 15
PROCEDURES FOR TOXIN IDENTIFICATION TESTS

Animal Number	Supernatant, Centrifugalized or Filtered Culture Fluid	Antitoxin
Guinea-pig 1.....	1 to 2 c.c. subcutaneously	0.5 to 1 c.c. of antitoxin for <i>B. botulinus</i> type A 0.5 to 1 c.c. of antitoxin for <i>B. botulinus</i> type B
Guinea-pig 2.....	1 to 2 c.c. subcutaneously	
Guinea-pig 3.....	1 to 2 c.c. subcutaneously	
Mouse 1.....	0.1 c.c. intraperitoneally	0.5 c.c. of antitoxin type A 0.5 c.c. of antitoxin type B
Mouse 2.....	0.1 c.c. intraperitoneally	
Mouse 3.....	0.1 c.c. intraperitoneally	

The toxins were mixed with the antitoxin in small test tubes and the mixtures were kept at room temperature for from one-half to 3 hours, depending on the number of samples. The toxin-antitoxin tests have been conclusive with cultures containing at least 1 to 10 minimal lethal doses per 2 c.c. In the majority of experiments, the control animals inoculated with the toxin alone succumbed a few hours earlier than those inoculated with the toxin and nonspecific antitoxin. It should be emphasized that inconsistent neutralization tests were invariably repeated either with filtered toxins or by feeding the latter to animals which had received subcutaneously 0.5 to 1.0 c.c. of antitoxin. A limited number of toxins have been encountered³² in which all 3 animals succumbed with symptoms of botulism or, if surviving, apparently incompletely protected guinea-pigs showed symptoms of intoxication. When this occurred, the experiment was repeated by inoculating a series of 5 animals. The first 3 were treated as shown in table 15, while the fourth received, besides the toxin, 1 c.c. each of antitoxin for *B. botulinus* type A and type B (1 c.c. of polyvalent antitoxin was used if available). The fifth guinea-pig

³¹ Determined according to the method of Bengtson.

³² The observations made in the laboratory have independently been confirmed by George E. Coleman of Santa Barbara.

received from 1 to 2 c.c. of filtrate or culture fluid which had previously been boiled for 10 minutes. The presence of a mixture of type A and B toxin and the presence or absence of heat resistant poison was demonstrated by this procedure. That this may be responsible for apparently equivocal results has been further proved by the isolation of *B. botulinus* type A and B from 2 different soil enrichment cultures.

Centrifugalized cultures producing tetanus were filtered; one observation had shown that this disease followed the inoculation of tetanus spores which had not been sedimented in the course of the centrifugalization. Some filtrates provoked symptoms of tetanus, and the test for the presence or absence of *B. botulinus* toxin was accomplished by mixing the filtrates with 1,000 units of commercial tetanus antitoxin or by feeding the suspected cultures to properly immunized guinea-pigs. A few toxic cultures could not be identified by the antitoxin-neutralization or feeding tests. Experiments are now in progress to determine the relationship of these toxic cultures to the recently described anaerobe, isolated by Miss Bengtson from the larvae of *Lucilia Caesar*.³³

In conclusion, it must be emphasized that toxic enrichment cultures should be considered positive for *B. botulinus* only when a decisive toxin—antitoxin reaction is obtained, or when the enrichment cultures provoke classical intoxications on feeding, or when the specific organism is isolated in a pure state.

ENRICHMENT OF WEAKLY TOXIC CULTURES

It has been pointed out that enrichment cultures may frequently contain less than one minimum lethal dose per 2 c.c. The inoculated guinea-pigs may exhibit for several days characteristic symptoms of botulism in the form of flabby abdominal muscles, striking emaciation, etc., and finally either recover or succumb to secondary infections. In the latter instance, the fairly characteristic necropsy findings are masked by a diplococcus, a *B. bronchisepticus* pneumonia or a generalized paratyphoid infection. At the beginning of the investigation an effort was made to produce a stronger toxin, by transplanting various amounts of the unheated or heated original enrichment cultures into flasks containing 100 c.c. of fresh sterile beef heart digest mediums and incubating again for 10 days at 35 C. This procedure had been used by Burke⁸ with some success, but has not proved satisfactory in this laboratory. Transplants from cultures in van Ermengem mediums were invariably nonpoisonous, while the toxicity of beef heart cultures was only slightly enhanced over the original culture. Various modifications

³³ Two series of experiments have shown that the toxin of the anaerobe isolated by Bengtson is produced in enrichment cultures and in the presence of soil bacteria. It is therefore not unlikely that toxin of some of the enrichment cultures which could not be neutralized by a polyvalent *B. botulinus* antitoxin belong to the group of the *Lucilia Caesar* poisons. This point can only be decided when a suitable antitoxin against the latter organism is available.

of heating the original culture at 80 to 100 C. were tried, but the toxicity was not appreciably increased. In fact, it was found advisable to prepare fresh cultures with the original soil or vegetable samples. This method has distinct advantages, namely: 1. It serves as a control for the original test. 2. It may yield at times more toxic cultures than the previous examination, which can be identified by an antitoxin—toxin test. 3. It may, furthermore, furnish cultures excellently suited for the isolation and purification of *B. botulinus* where this was not formerly possible.

These tests, which were conducted at monthly intervals, served as sterility controls for the laboratory procedures, assured the workers

TABLE 16
FINDINGS IN EXPER. 19

Number	Specimens	1st Culture: 2 c c	2d Culture: 2 c c
(1)	Beets, Kansas (149).....	Symptoms, death 48 hours	Death, 48 hours, type A
(2)	Soil, cloverfield.....	Death on 5th day paratyphoid infection	Nontoxic
(3)	Soil, cornfield.....	Death in 16 hours	Death, 16 hours, type B
(4)	Soja beans.....	Symptoms, recovered	Symptoms, death 12th day
(5)	Beet tops, Louisiana.....	Symptoms	Death 4th day, type A
(6)	Moldy hay.....	Nontoxic	Nontoxic
(7)	Rice straw.....	Symptoms	Death, 48 hours, type A
(8)	Soil, Pennsylvania.....	Symptoms	Nontoxic
(9)	Soil, pasture.....	Symptoms	Death, 18 hours, type A
(10)	Soil, ryefield.....	Symptoms	Death, 48 hours, type B
(11)	Garden soil.....	Death, 5th day	Death, 48 hours, type A
(12)	Soil, horse pen.....	Death, vibriion sep- tique infection	Nontoxic
(13)	Soils, San Bernardino.....	Symptoms	Death, 48 hours, type B
(14)	Soil, Pasadena.....	Death in 72 hours	Marked symptoms
(15)	Dirt, horse corral.....	Nontoxic	Nontoxic
(16)	Soil, hog pen.....	Death, 4th day	Death, 48 hours, type B
(17)	Decayed alfalfa.....	Death in 24 hours, pneumonia	Nontoxic
(18)	Virgin soil.....	Death in 36 hours, vibriion septique	Nontoxic
(19)	Soil, Alleghany mountains.....	Death in 36 hours, tetanus	Nontoxic
(20)	Potato pits, Yakima.....	Death in 24 hours, type A	Nontoxic

that laboratory contaminations had been eliminated and provided data which indicated that the spores of *B. botulinus* were unevenly distributed in the specimens. These statements are best illustrated by the presentation of one test.

Exper. 19.—Specimens which produced unsatisfactory enrichment cultures on account of prolonged incubation or storage and those which were frankly nontoxic were repeatedly cultivated in beef heart digest broth and incubated for 10 days at 35 C. The findings are presented in table 16.

The value of making repeated cultures on doubtful specimens is fully established by the data shown in table 16. It is unnecessary to

state that several hundreds of identical tests have been carried out with similar results. Occasionally, as shown under No. 20, a specimen produces an original toxic culture, but on retesting it is found to be nontoxic. Cultures of such specimens have been made a third and fourth time, and in this manner evidence is procured which indicates that heat resistant spores may be quite unevenly distributed in soil or vegetable specimens. In order to prove the presence or absence of *B. botulinus* spores, repeated cultures should be regularly made from the original specimens, and doubtful cultures should be tested by this procedure instead of transplanting enriched cultures to fresh mediums.

Based on the experiments described in the foregoing paragraphs, the following method is now used for the enrichment and demonstration of *B. botulinus* in specimens of soil and its products:

THE METHOD EMPLOYED IN THIS LABORATORY

Twenty to 50 gm. of soil or vegetables or fruit peelings, etc., are placed in a 250 cc culture flask (No. 2221, Arthur H. Thomas Catalogue) and thoroughly suspended or mixed in sterile salt solution (P_H 7.0). Moist soil is previously ground in a mortar. Hay and dry vegetable specimens are finely chopped and then soaked for several hours in salt solution. The suspensions, etc., are heated in a water bath for 1 to 2 hours at 60 to 70 C. or 1 hour at 80 C. While warm, the suspensions are mixed with 100 c.c. freshly boiled beef heart peptic digest liver broth with a reaction of P_H 7.2-7.4. The fluid is either covered with petrolatum or liquid petrolatum to avoid evaporation, or the flasks are closed by a sterile rubber stopper provided with a piece of glass tubing. The container is rapidly exhausted and the glass tubing is allowed to seal itself off by placing it in the middle of a small bunsen flame. The cultures are incubated for 10 days at 35 C. A small sample of the supernatant fluid is then removed and centrifugalized for 1 hour at high speed. Two c.c. of the clear fluid are injected subcutaneously along the median line of the abdomen of guinea-pigs weighing 250 to 300 gm. The symptoms of the animals are recorded by daily inspections, and the dead guinea-pigs are carefully necropsied. Cultures which are fatal to guinea-pigs are further tested by a toxin-antitoxin test with type A and B antisera. When the cultures contain less than one M L D per 2 c.c. cultures of the original material are made a second, even a third and fourth time. Centrifugalized cultures are only filtered through small Mandler candles provided the inoculated guinea-pigs succumb to mixed anaerobic infections or present symptoms of tetanus. Such fluids may be tested by feeding immunized and nonimmunized guinea-pigs with heated and unheated cultures. The latter procedure is, however, less dependable than subcutaneous inoculation. Filtered or centrifugalized cultures which are known to be free from concomitant anaerobes may be "typed" on mice by intraperitoneal inoculations. Specimens are considered positive for *B. botulinus* only when a decisive antitoxin-toxin test has been obtained or the organism has been isolated in pure culture.

In order to avoid laboratory contaminations, the culture mediums, salt solution, flasks, etc., should be autoclaved at 18 pounds' pressure for 1 hour and resterilized for the same period after an incubation of from 5 to 14 days. Contaminated glassware, cultures, etc., are treated similarly for 3 hours at

18 pounds' pressure. The working benches, trays, balances, etc., are cleansed with 50% dilution of a compound solution of cresol or warm 10 to 20% formaldehyde solution. Inexperienced workers should question their results until they have eliminated the danger of laboratory contaminations by continuous, painstaking vigilance and repeated control examinations.

THE ISOLATION AND PURIFICATION OF *B. BOTULINUS* FROM TOXIC SOIL ENRICHMENT CULTURES

It has been pointed out that highly toxic cultures may be obtained by proper enrichment methods, although it is impossible to demonstrate *B. botulinus* by the customary procedures employed for the direct primary isolation of anaerobes. The statement made by a group of workers that they "never failed to obtain the organism in pure culture from the broth in which a virulent toxin had developed," applies in all probability to the enrichment cultures obtained from spoiled food in which *B. botulinus* was the main and often the only spore-bearing anaerobe.

In the course of a number of preliminary tests during which small amounts of toxic enrichment cultures were distributed by dilutions into deep liver agar shake tubes, it was noted that only those samples which were fatal to guinea-pigs in less than 16 hours (2 c.c. subcutaneously) were of sufficient promise to devote time and material for isolation. This process of selection reduced the specimens suitable for isolation to less than 10% of the cultures, but even the agar columns of these samples rarely presented colonies in sufficient numbers or of characteristic structure to permit of their rapid and conclusive identification as those of *B. botulinus*. These failures led to a number of experiments with selective mediums. Attempts were made to enrich overwhelmingly the *B. botulinus* present in the original enrichment culture by successive passage through mediums of various composition and reaction. Invariably, a substratum favoring *B. botulinus* encouraged also the associated proteolytic anaerobes. Certain suggestive leads have been encountered in the course of these studies, and it is not unlikely that a selective medium may be developed in the future. As it was, however, imperative to isolate in the shortest possible time a large series of strains of *B. botulinus*, it was found necessary to make use of the exceptionally marked heat resistance of the spores of *B. botulinus*. Two procedures have yielded a number of successful isolations, namely:

1. The enrichment cultures (10 days at 35 C.) are either heated at 80 C. for 1 hour or boiled for 10 minutes. The sediment of the cultures is sealed in 1 to 2 c.c. amounts in small glass tubes and subjected to the heat. Small

samples of the heated specimens are immediately plated in sheep blood agar, or sown into deep liver agar tubes or enriched for from 24 to 120 hours in beef heart peptic digest broth and then cultivated in deep agar. The latter procedure may be repeated leading frequently to enrichments suitable for isolations.

2. Twenty to 50 gm. of soil, which had previously been found to contain a large number of *B. botulinus*, are suspended in an equal amount of salt solution. The suspensions are exposed to steam in an Arnold sterilizer for from 1 to 1½ hours. The heated soil specimens are then cultivated in beef heart peptic digest broth according to the customary procedures. Subcultures are made daily in deep agar shake tubes. Several strains have been isolated by this method; in the heated cultures *B. botulinus* and occasionally *B. sporogenes* and *B. welchii* are the only surviving and freely growing anaerobes.

As a part of the practical control of botulism in the United States rests primarily on an accurate knowledge of the maximum resistance of the spores to heat, the use of these procedures appears justified. It may be assumed that the boiling or steaming selectively eliminated the non-resistant spores and in this manner furnished strains of maximum resistance. For studies dealing exclusively with the biology and the distribution of various types of *B. botulinus* in nature these methods are, therefore, not practical. It has, for example, been impossible to obtain toxic enrichment cultures by boiling or steaming soil samples which contained the spores of *B. botulinus* type B. Furthermore, many interesting problems dealing with the distribution of the various mutants of the genus *Botulinus* cannot be solved by "heating methods," and, therefore, no effort should be spared to develop a selective enrichment medium. It is self evident that the procedures mentioned can also be applied to the isolation of *B. botulinus* from the intestinal contents and the decomposed viscera of man and animals. Subsequent paragraphs will consider the details to be observed in handling these specimens.

One of the writers (K.F.M.) has used, since 1907, the deep agar shake method for the primary isolation and purification of anaerobes. Various modifications and improvements have recently been summarized in several papers by H. H. Heller.²¹ The technical principles mentioned in these communications have been followed in the isolation of *B. botulinus* and need no additional comment. Peptic digest or veal infusion glucose (0.5%) agar has frequently been used for primary isolation instead of liver agar. In searching the agar column of the cultures for characteristic colonies, a Mayer's binocular dissecting microscope has been employed and found invaluable in saving time and eyesight. In this connection, it should be stated that only the mass

colonies which correspond in general appearance to those described by Burke were considered characteristic. Experiences with over 100 cultures of *B. botulinus* isolated from various sources, which will be reported in another paper, indicate that the shape and the thickness of the colonies is sufficiently constant to enable detection in crowded mixed cultures. It is customary to use a fairly dry 2% agar and to incubate the various seed and dilution tubes at 28 to 35 C. for at least 10 days. The heating injures the spores considerably and delayed germination is frequently observed.

Laboratory workers who intend to isolate *B. botulinus* from mixed enrichment cultures should thoroughly familiarize themselves with the appearance of the mass colonies of authentic, recently isolated strains. In particular, the shape, size and texture of such colonies should be studied in deep agar of various composition and colloid content. This recommendation is made because certain toxic and pure stock cultures, circulating in this country, produce in deep agar atypical mutation colonies. These strains of *B. botulinus* convey an entirely wrong impression of the colony characteristics and, when adopted as a standard, prevent the inexperienced from selecting and fishing *B. botulinus* colonies. It is naturally quite possible that in this laboratory a number of toxic strains of *B. botulinus* have not been recognized and isolated on account of the adopted conservative policy of picking only disk-shaped colonies. In this connection, it must be admitted that even the latter type is not absolutely specific for *B. botulinus*. A number of anaerobes forming disk colonies not unlike those of *B. botulinus* have been found in soil enrichment cultures. These bacteria, when isolated and purified, resemble, morphologically and biochemically, *B. botulinus*, but they fail to produce a toxin. Experiments with these strains will be reported elsewhere more in detail. Another condition deserves attention: Crowded, deep agar shake tubes frequently reveal small, rather transparent disk colonies with heavy polar tufts. These colonies are usually formed by various terminal end spore bearers and are easily mistaken by the beginner for those of *B. botulinus*. It can be said without fear of contradiction that considerable experience is needed to be thoroughly familiar with the various types of disk colonies characteristic of *B. botulinus*.

The colonies which are considered typical are picked by means of fine capillary tubes and are transferred to a series of deep agar or beef heart peptic digest broth tubes. As soon as toxin-antitoxin tests on mice and microscopic examinations have proved the nature of the

isolated colony and confirmed the type of the original enrichment culture, the process of purification is undertaken. Aerobic contaminations have usually been eliminated in the course of repeated heatings so that cultivation of young beef heart peptic digest broth cultures in deep agar tubes furnishes pure cultures in 95% of the instances. Cultures under inspection for purity are kept in beef heart broth and veal infusion digest broth for from 4 to 6 weeks at 28, 37 and 56 C. Shake cultures in deep agar are made at regular intervals; isolated colonies are picked and transferred to beef heart tubes. During the last year successive plating in sheep blood peptic digest agar with the aid of an anaerobic jar method to be described by C. C. Dozier, or according to the inverted plate method of Krumwiede and Pratt, or surface plating on milk agar, has been used to verify the purifications accomplished by the deep culture method. Several cultures have been further purified by Barber's microscopic method of single bacillus isolation. The employment of the latter procedure is, however, unnecessary. Several hundred different anaerobic cultures have been isolated and purified in this laboratory by the deep culture method, and it has been noted that the contaminations of properly purified cultures were either aerobes, as molds, cocci and subtilis types or adventitious anaerobes due to improperly sterilized mediums. The rigid examination of carefully purified cultures of *B. botulinus* during the last 2½ years has not shaken the original conclusion that the deep culture method, occasionally combined with the blood agar or milk agar plating method, is the most practical and reliable means for the isolation and purification of these organisms.

It is customary in this laboratory to preserve one culture of the original strain of anaerobe after purification in sealed tubes of beef heart peptic digest broth. In case of accidental contamination of the stock culture or for future reference these tubes saved time, worry and labor.

In conclusion, it should be emphasized that the primary isolation of *B. botulinus* from toxic soil, vegetable or manure enrichment cultures is only possible when the original specimen contains a fairly large number of heat resistant spores. Based on numerous observations, it is recommended that in enrichment cultures containing on the 10th day of incubation at 35 C. approximately 1,000 to 10,000 M L D isolation of the organism in pure culture should be attempted by the deep agar method. The results when the degree of toxicity is below this point are too generally negative to warrant the procedure.

EXAMINATION OF SUSPECTED FOOD

Before discussing the methods adapted to the examination of suspected food, it is deemed advisable to consider the principles involved and to indicate the great importance of this phase of technical mycology. The demonstration of *B. botulinus* and its toxin in preserved or canned vegetables, meats, etc., may have a twofold purpose, namely: 1. It may confirm a provisional clinical diagnosis and from a medicolegal standpoint explain the true nature of the intoxication, or (2) it may supply important information relative to the type of *B. botulinus* antitoxin which may be useful in the treatment of the disease. It is evident that in every suspected outbreak of botulism a toxicologic examination by inoculation or by feeding of the suspected food to guinea-pigs and mice should be carried out, and no effort should be spared to procure specimens suitable for this purpose. Little accurate knowledge relative to the average toxicity of spoiled canned foods is available. Future toxin tests should carefully determine the average number of lethal doses per 1 c c of vegetable juice. In case a specimen is nontoxic, cultures should be made. Occasionally the results may be vitiated by the fact that suspected foods are collected after an exposure to air and light for several days. Furthermore, that portion of the vegetables available for examination may have been subjected to more heat in the course of preparation than that consumed by the patients, or the sample may have been made susceptible in transit to factors which destroyed the toxin. Under these circumstances, what is apparently a nontoxic specimen will often furnish toxic enrichment cultures, or numerous typical colonies will develop in specimens prepared by the deep agar method.

Old and recent observations have shown that the development of *B. botulinus* and the generation of its toxin in home and commercially canned vegetables and fruits may be very irregular. It has been pointed out in another paper that the occurrence of demonstrable toxin in canned products is the exception rather than the rule. For example, Koser, Edmonson and Giltner ³⁴ have shown that the contents of only 6 of 82 defective containers of spinach were found to be toxic when fed to guinea-pigs, while workers in this laboratory made similar observations on home canned string beans and asparagus. The content of one jar may be toxic, while 10 or 12 others packed at the same time and under identical conditions may be spoiled and nontoxic, or may be

³⁴ Jour. Am. Med. Assn., 1921, 77, p. 1250.

normal in appearance, and yet on enrichment may furnish a culture of *B. botulinus*. The present day information relative to the ability of *B. botulinus* to survive or to germinate in vegetables and fruits is incomplete, and a systematic search for this organism and its toxin in home and commercially canned food by direct or enrichment cultures appears imperative for the following reasons:

1. Nothing is known in regard to the distribution of *B. botulinus* spores in under-processed canned foods, whether spoiled or sound. The cultural methods employed by Weinzirol,³⁵ Cheyney,³⁶ and the Micro-biological Laboratory³⁷ of the Bureau of Chemistry are inadequate. The samples chosen for their tests were too small and no special anaerobic methods or mediums were employed. Until the possibility of botulinus spoilage had been recognized, processing procedures were frequently used which did not destroy the spores of *B. botulinus*. In the majority of instances the few surviving spores were seriously injured and germination failed to take place, or it was retarded for months. Larger samples of suspected food material, or cans which present no evidence of bacterial activity must, therefore, be enriched in suitable anaerobic mediums. These recommendations apply to commercially as well as to home canned foods; particularly in the latter products absolute sterility is usually impossible on account of the customary processing with boiling water. It would, therefore, be of great value to know accurately the average percentage of containers in households which hold viable spores of *B. botulinus*. With such information available, the factors preventing or enhancing the germination and toxin production could be studied more intelligently and the question, Why is botulism due to canned vegetables, etc., an acquisition of recent years? could probably be answered by reference to scientific facts instead of assumptions.

2. Direct cultures by the deep and plate agar methods should be made of toxic and nontoxic spoiled canned vegetables and fruits in order to determine the bacterial flora. By this procedure, the relative proportion of *B. botulinus* to other anaerobes or aerobes could be perfectly estimated and the influence of the latter organisms on the toxin production be established more accurately than has hitherto been attempted. Furthermore, these tests could serve as valuable contributions to the study of anaerobic spoilage occurring in canned products

³⁵ Jour. Med. Res., 1919, 39, p. 349.

³⁶ Ibid., 1919, 40, p. 177.

³⁷ Jour. Am. Med. Assn., 1920, 74, p. 1220. Also reference 34.

and would either support or refute the contention of some workers who attribute great practical importance to the constant association of other bacteria with botulinus organisms. Numerous observations made in this laboratory have shown that commercially packed spoiled and toxic spinach contains no other organism than *B. botulinus*, and symbiosis is obviously not a prerequisite for toxin production in this vegetable. However, it has been fully proved that any lot of canned food which shows an appreciable percentage of spoilage is potentially dangerous, but no laboratory has as yet determined the percentage of containers of such lots which may harbor viable spores of *B. botulinus*. Until such tests have been carried out every can or jar of a batch or lot showing even less than 1% spoilage must theoretically be considered suspicious. It has been definitely proved that the presence of *B. botulinus* in canned food is always indicative of poor sterilization, and a few recent investigations have disclosed that not one container, but usually the entire lot or pack, has been improperly processed.

It will be evident from this brief discussion that the bacteriologic examination of suspected food, whether spoiled or sound, necessitates the use of proper procedures. The methods which have been found dependable for this purpose are, therefore, presented in detail.

A. Tests for Toxicity of Foods.—1. A 10 cc sample or the entire available amount of vegetable juice or brine is either centrifugalized or is filtered through paper and then centrifugalized. In some cases, the clarified specimens may still be contaminated with aerobic cocci and rods; filtration through a Mandler candle is then recommended. A portion of the filtrate or centrifugalizate is boiled for 30 minutes. Two mice each are inoculated intraperitoneally with 0.5 cc of unheated or boiled juice. Guinea-pigs weighing not more than 300 gm. are similarly treated with amounts of from 1.0 to 2.0 cc. The animals inoculated with the unheated material will succumb in from 4 to 72 hours, provided the food specimen contained the toxin of *B. botulinus* in sufficient concentration to produce symptoms or death in human beings, when ingested. It is self-evident that the animals injected with the heated specimens should remain alive, provided the specimen contains only the toxin of *B. botulinus* and no metallic poisons. For confirmation of the inoculation tests, it has frequently been found practical to feed samples of the suspected food to immunized and nonimmunized guinea-pigs.

2. In case of meat or vegetable foods, which contain little fluid, three procedures may be followed, namely: (a) A portion of the specimen may be administered by forced feeding to guinea-pigs, or (b) mixed with the regular feed offered these animals, or (c) a fairly large sample or the entire available remnant of the food may be ground in a mortar and then extracted for from one to two hours at room temperature with sterile salt solution. The extract is centrifugalized and the supernatant fluid is tested either directly or after filtration by the inoculation of mice and guinea-pigs, as outlined under (1), while the sediment is preserved for subsequent tests to be outlined under B.

3. Unopened, but suspected, tins or glass containers are thoroughly scrubbed with soap in hot running water, the top and sides dipped in a warm 20% formaldehyde solution and the latter permitted to act for from 10 to 15 minutes. The top or lids are flamed just before being opened. A "radial" can opener sterilized in oil or in the autoclave is used, cutting a circular opening of from 2 to 3 inches in diameter. A 10 to 15 c c portion of the juice or brine is pipetted into centrifuge tubes; with the aid of forceps, which have been sterilized in liquid paraffin oil of a temperature of 180 C., specimens varying from 25 to 250 gm. are transferred to sterile Blake bottles for extraction or cultures. The opening of the container is covered with sterile paper and the can stored in the icebox. The unheated and heated centrifugalized liquid portion or extract of the food is tested on mice or guinea-pigs as stated under (1).

4. The type of *B. botulinus* toxin present in foods is determined by the inoculation of 3 guinea-pigs or 3 mice, one with the centrifugalized or filtered juice or extract alone, one previously inoculated with from 0.5 to 1.0 c c type A antitoxin and another with type B antitoxin. In order to approximate the correct dose of toxin, serial inoculations of 3 mice each, as recommended by Bengtson³⁸ are sometimes necessary. The inoculations can be made as shown in table 17.

TABLE 17
SERIAL INOCULATIONS OF MICE TO DETERMINE CORRECT DOSE OF TOXIN

A	{	Mouse 1—1	c c of juice, brine or extract
	{	Mouse 2—1	c c of juice, brine or extract and 0.5 to 1.0 c c of type A antitoxin
	{	Mouse 3—1	c c of juice, brine or extract and 0.5 to 1.0 c c of type B antitoxin
B	{	Mouse 4—0.5	c c of juice, brine or extract
	{	Mouse 5—0.5	c c of juice, brine or extract and 0.5 to 1.0 c c of type A antitoxin
	{	Mouse 6—0.5	c c of juice, brine or extract and 0.5 to 1.0 c c of type B antitoxin
C	{	Mouse 7—0.1	c c of juice, brine or extract
	{	Mouse 8—0.1	c c of juice, brine or extract and 0.5 to 1.0 c c of type A antitoxin
	{	Mouse 9—0.1	c c of juice, brine or extract and 0.5 to 1.0 c c of type B antitoxin

The examination of feeds involved in outbreaks of fowl botulism or "forage poisoning" in horses has been conducted according to the same principles. Large samples of the feed, varying from 2 to 20 lbs. are soaked in sterile tap water for from 24 to 48 hours at room temperature. The fluid is recovered from the plant material by means of a meat press, strained through paper and tested either unfiltered by feeding or filtered by subcutaneous inoculations on guinea-pigs. "Mass enrichment" cultures are also made with the soaked and heated feed samples. As the workers of this laboratory have thus far been unable to detect the toxin of *B. botulinus* in moldy hay, grain or silage suspected of causing botulism in horses or cattle, it is impossible to conclude that the methods employed are really dependable.

B. Isolation of B. botulinus from Suspected Food.—Two procedures should be used: (1) deep agar shake cultures, and (2) enrichment "mass cultures" with heated and unheated specimens.

(1) The sediment of the centrifugalized juice or extract is divided into 2 portions, and 1 is heated in a thin, narrow tube for from 30 to 60 minutes at 70 C. Adequate samples of the heated and unheated specimens are distributed in melted liver peptone or peptic digest agar. It is advisable to prepare triplicate sets and to incubate one at 35 C., one at from 25 to 28 C. and

³⁸ Public Health Reports, 1921, 36, p. 1665.

one at 55 C. This method permits a quantitative estimation of the number of viable organisms or spores per 1 cc of juice or brine. By making dilutions in broth and transferring 1 cc amounts to from 10 to 15 cc of melted agar in deep tubes or to warm blood agar tubes, which are poured into plates, isolated colonies are usually obtained. The inoculated tubes or plates should be kept under observation for at least 10 to 20 days. Characteristic disk-shaped colonies are readily counted and frequently permit the isolation of a pure culture.

(2) The sediment used for shake cultures or a 20 to 250 gm. sample or the entire available remnant of the food is suspended in appropriate amounts, heated for from 1 to 2 hours at 60 to 70 C. and then mixed with large amounts of freshly boiled beef heart peptic digest liver broth. In case the specimen is very acid or distinctly alkaline, as for example string bean salad prepared with vinegar or olive brine, it is advisable to neutralize the salt suspension with sodium carbonate or dilute lactic acid. Observations have shown that the heating of acid or alkaline specimens either destroys or injures the viable spores, and nontoxic enrichment cultures are sometimes obtained. Unheated specimens should also be tried in case of unsatisfactory nontoxic cultures.

The enrichment cultures are incubated for from 5 to 10 days at 35 C. and then tested for *B. botulinus* toxin. The principles followed for the isolation of *B. botulinus* from toxic cultures is the same as described in previous chapters. Purified cultures are identified by toxin-antitoxin tests. It is self-evident that the type of the isolated strain should correspond with that previously established for the toxin of the suspected food, juice or extract.

In order to stimulate a further investigation of suspected foods, either commercially packed or home canned products, etc., the observations made in this laboratory with the foregoing methods during the last two years are summarized in table 18.

EXAMINATION OF CLINICAL MATERIAL

In a previous communication,³⁹ the isolation of *B. botulinus* from the stools of patients with clinical cases has been described. Fecal specimens from 4 clinical cases (Reports 3, 4, and 63) obtained from 3 different outbreaks contained *B. botulinus* type A on the 6th, 7th, 11th, and 12th day, respectively, after the consumption of the causative meal. One specimen procured from a case on the 31st day (Report 63) and another from a case on the 26th day of illness (Report 81) were negative. Over 80 stool specimens of people who had ingested raw vegetables and fruits purchased in the open market proved negative for *B. botulinus*, even though such foods had demonstrated on repeated examinations the presence of botulinus spores.

The method employed is briefly as follows:

Stool specimens as large as possible are diluted with salt solution until the formed portions are finely divided. The emulsions are placed in 250 cc culture flasks and heated for one hour at from 60 to 70 C. They are then

³⁹ *Ibid.*, 1921, 36, p. 1313.

TABLE 18
RESULTS WITH PRESERVED FOODS

Num- ber of Sample	Food or Food Products	Origin	Condition of Con- tainer	Physical Examination of Product		Toxin Test	Cultural Tests		B. botu- linus
				Odor	Appearance		Aerobes	Anaerobes	
53 I a	Pickled ripe olives	L, Cal- ifornia	"Swell"	Normal	Soft and dis- colored turbid brine	Negative	Spore bearing and gram-negative cocci	B. botulinus in enrichment also terminal end spore bearer	Type A
56 B	Pickled ripe olives	L, Cal- ifornia	"Springer"	Abnormal	Soft and tur- bid brine	Negative	Spore bearing and gram-negative cocci	B. botulinus in enrichment also terminal end spore bearer	Type A
81 B	Pickled ripe olives	O, Cal- ifornia	"Swell"	Bad	Soft	Negative	Rods and cocci	B. botulinus in enrichment B. sporogenes and term- inal end spore bearer	Type B
105 a	Home canned asparagus	Washing- ton	Bulging lid	Foul	Disintegrated stalk, turbid liquor	Positive M L D 1:50,000	Thermophilles and gram-positive rods	Pure B. botulinus	Type A
105 b	Home canned asparagus	Washing- ton	Leaking	Foul	Disintegrated stalk, turbid liquor	Positive M L D 1:10,000	Negative	Pure B. botulinus	Type A
118	Commercially packed asparagus, 2 cans	California	"Swell"	Sour	Soft stalk	Negative	Thermophilles	Enrichment pure B. botu- linus	Type A
120 II	Commercially packed spinach, seized	Washing- ton	Hard swell	Cheesy	Soft mushy	Positive M L D 1:10,000	Sterile	Pure B. botulinus direct and enriched	Type A
120 III	Commercially packed spinach, seized	California	Hard swell	Cheesy		Positive	Sterile	Pure B. botulinus direct and enriched	Type A
114 C	Home canned fried chicken	California	Flat lid but leaking	Repulsive	Soft, but no liquor	Negative	Cocci and Molds	Enrichment B. botulinus and B. sporogenes	Type A
220	Home canned string beans, 2 cans	Oregon	Flat	Normal	Firm	Negative	Spore-bearing rods	Direct terminal and spore bearer, enriched pure B. botulinus	Type A
221	Home canned corn	Idaho	Bulging lid, leaking	Sour	Firm, dry, no liquor		Fine gram-positive rods	Direct pure B. botulinus	Type A
222	Five unprocessed "bulk olives"	California	Gas, turbid brine sediment, soft olives	Extract positive Brine nontoxic	Variety of rods, cocci, etc.	B. botulinus, B. spor- ogenes, terminal end spores	Type A

mixed with beef heart peptic digest liver broth, exhausted and incubated as previously described. Tests for toxin and isolation of *B. botulinus* in pure culture are made according to the methods developed in this laboratory.

Bengtson³⁸ has suggested the intraperitoneal inoculation of white mice with the citrated blood of persons suffering from botulism in order to establish a differential diagnosis, or to determine the type of intoxication. Kob,⁴⁰ in 1905, reported that the serum from a child collected on the 9th day after eating poisonous meat caused typical symptoms when injected into white mice intraperitoneally, and recently Semerau and Noack,⁴¹ in Strassburg, claimed to have demonstrated by the inoculation of guinea-pigs the presence of toxin in the serum of 4 patients on the 4th, 6th, 9th, 16th and even on the 25th day after the consumption of the poisonous food. In America, this procedure has been tried on only one case (Report 80) with a negative outcome. These tests are of considerable clinical and scientific importance and should by all means be repeated when the occasion arises.

TABLE 19
B. BOTULINUS IN HUMAN NECROPSY SPECIMENS

Outbreaks	Location	Specimens Examined	Toxin Tests with Salt Extracts	Positive Cultures	Type of <i>B. botulinus</i>
Report 7	Richmond, Calif.	Spleen, liver, lungs, kidneys, colon and upper ileum	Negative	Negative	
Report 15	San Rafael, Calif.	Spleen, liver and colon	Negative	Negative	
Report 68	Florence, Ariz.	Spleen, jejunal content, jejunal wall, brain	Negative	Intestinal wall	B
Report 98	Healdsburg, Calif.	Spleen, liver, transverse colon	Negative	Liver, transverse colon content and wall	A

EXAMINATION OF NECROPSY MATERIAL

In connection with medicolegal questions, systematic necropsy cultures should be carried out by means of dependable procedures. Revived interest in the relation of *B. botulinus* to certain animal diseases necessitates likewise the application of definite bacteriologic methods. We shall describe the procedures employed in the study of (a) human, (b) chicken and (c) horse and cattle necropsies.

(a) *Human Necropsies*.—Specimens from 4 necropsies have been examined. The pieces of spleen and liver were thoroughly sterilized by immersion in hot paraffin oil (180 C.). Samples varying from 25 to 150 gm. were ground with sand and thoroughly emulsified in salt solution. Intestinal chyme or dry fecal pellets adherent to the mucosa of the small and large intestines and portions of the intestinal mucosa were emulsified in salt solution. The emulsions were, in 2 instances, tested for toxins of *B. botulinus* and then heated for one hour at 70 C. and mixed with beef heart peptic digest liver broth. The enrichment

⁴⁰ Med. Klin., 1905, 1, p. 84.

⁴¹ Ztschr. f. klin. Med., 1919, 88, p. 304.

cultures were tested after an incubation period of 10 days at 37 C. *B. botulinus* was readily isolated from these cultures. The results of these studies are shown in table 19.

Attention is called to the absence of *B. botulinus* in 4 spleen cultures. These observations are quite in harmony with the findings on experimental animals. Guinea-pigs which succumb to botulism following the ingestion of large amounts of detoxified spores of *B. botulinus* may furnish sterile splenic cultures, while the mesenteric lymphnodes, the liver and bonemarrow give positive enrichments. The demonstration of *B. botulinus* in the stools of patients with clinical cases suggests an examination of the colon and the rectum. The results obtained in a recent outbreak encourages the belief that a quantitative study

TABLE 20
B. BOTULINUS AND ITS TOXIN IN CHICKEN NECROPSY SPECIMENS

Report*	Location	Specimens Examined	Toxin Tests with Extracts	Positive Cultures	Type of <i>B. botulinus</i>
16	Saratoga, Calif.	Spleen, liver, crop, gizzard, kidneys, lungs, intestines, 6th day of disease	Negative	Caece, but not small intestine or colon	A
19	Los Angeles, Calif.	Spleen, liver, gizzard, crop, small and large intestines	Crop content negative	Spleen, liver, crop, gizzard, small and large intestines	A
26	Klamath Falls, Ore.	Spleen, liver, gizzard, crop, intestines	Crop content positive, but not intestines	Crop content only	A
51	Tieton, Wash.	Crop, gizzard, spleen	Crop content positive	Crop content only	B
53	Menlo Park, Calif.	Crops of 3 decomposed chickens, buried 5 days	Negative	Crops of all 3	A
92	San Jacinto, Calif.	Crops, gizzards, intestinal content, spleens and livers of 2 chickens	Crop of 1 chicken	Crops and contents of two, gizzard and intestines of one	A
105	Yakima, Wash.	Crop only	Positive	Crop	A
106	Glendale, Calif.	Crop, intestines and gizzard of one and crop content and wall of another	Negative	Crop content and wall of both	A
107	Fort Collins, Colo.	Crop content	Positive 0.01 c c for mouse	Crop and jar of corn	A

* The number refers to the reports mentioned in the paper; The Epidemiology of Botulism, to be published in a bulletin of the U. S. Public Health Service.

of such specimens may furnish valuable information as to the possible growth of *B. botulinus* in the intestinal tube. From the standpoint of early diagnosis, it is possible that the presence of the ingested toxin might be demonstrated in vomitus or contents of the stomach by the inoculation of laboratory animals with filtrates.

(b) *Chicken Necropsies.*—The importance of bacteriologic studies of cadavers of fowls has been discussed in previous papers. In a number of recent studies an attempt has been made to test the crop and intestinal content for the toxin of *B. botulinus* and to make cultures of the organs by means of enrichments. The entire content of the crop or gizzard is suspended in salt solution, thoroughly shaken and allowed to stand at room temperature for from 1 to 2 hours. The extract is filtered through paper and is either centrifugalized or passed

through a Mandler candle. Mice or guinea-pigs are injected with or without antitoxin. Cultures of the organs are prepared according to the procedures mentioned under (a). The results obtained by those methods are presented in table 20.

The demonstration of the toxin of *B. botulinus* in the crop has been possible in the cadavers of birds which have succumbed to the intoxication in from 24 to 48 hours. It is self-evident that the necropsies have been performed, as a rule, shortly after death. The excised tissues have been shipped separately in sterile containers. Toxin tests alone support the clinical diagnosis of botulism, while the isolation of *B. botulinus* from the crop or intestinal content lacks etiologic significance on account of the wide distribution of this organism in soil and its products with which chickens have intimate contact.

(c) *Necropsy Specimens of Horses and Cattle*.—In making cultures of organs or of the intestinal content of large domestic animals, painstaking attention has been paid to a thorough sterilization of the outside portion of the samples in order to avoid accidental contaminations. The tissues of 2 horses and 1 mule which succumbed to forage poisoning and of 2 cows which suffered from icterohemoglobinuria have been tested for toxin and cultures made by the methods described under (a) and (b). *B. botulinus* has been isolated from the intestinal content of the horses and the liver, mesenteric lymphnodes, etc., of 2 cows. The procedures have, therefore, proved satisfactory but have shown that *B. botulinus* is not infrequently a common inhabitant of the intestinal tract of domestic animals. In another paper it has been pointed out that an etiologic relationship of these organisms to the diseases or lesions from which they have been isolated is not proved. In future studies, therefore, an attempt should be made to demonstrate the toxin in the suspected feed or in the gastric content of the animals which exhibit symptoms of botulism.

SUMMARY

This paper describes the methods employed for the enrichment and demonstration of *B. botulinus* in specimens of soil and its products. The composition of the medium, the preparation and heating of the samples, the period of incubation and storage, the identification of the toxin and the isolation of the organism from toxic enrichment cultures are discussed and their value considered in the light of numerous experiments. It is emphasized that inexperienced workers should question their results until they eliminate the danger of laboratory contamination by continuous, painstaking vigilance, proper sterilization of the culture mediums, glassware, etc., and by repeated control examination.

The methods applicable for the examinations of suspected food, clinical and necropsy material are described in detail.

THE DISTRIBUTION OF THE SPORES OF *B. BOTULINUS* IN CALIFORNIA. II *

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In the course of an investigation of certain phases of botulism, it was realized that the prevention of this intoxication should not rest with an attempt to destroy the spores, but should go further and determine, if possible, the natural habitat of this formidable species. Such an inquiry, conducted in California, was not only imperative on account of the rather frequent occurrence of human and animal botulismus following the consumption of vegetable products grown and packed in this state, but appeared most promising on account of the preliminary studies conducted by G. S. Burke.¹ This worker found the spores of *B. botulinus* on apparently sound fruits, vegetables and feeds collected at widely separated localities in California. The organism was not in any way associated with active decay, although on one occasion a strain was isolated from the feces of a hog which had ingested spoiled, toxic peas four months previous to the collection of the manure specimen. This latter observation coincided with the report of Kempner,² who recovered, in 1897, a strain of *B. botulinus* from the intestinal content of a "normal hog" and indicated that at least in a small percentage of instances the spores, and perhaps the vegetative stages of this anaerobe may persist as saprophytes in the intestinal canal of animals.

The successful demonstration of *B. botulinus* on fruits, vegetables, etc., by Burke immediately suggested the existence of this anaerobe in the soil. From the facts available, however, it was by no means clear whether *B. botulinus* occurred only in cultivated regions or whether the bacillus was a part of the common soil flora and multiplied under natural conditions in forest and mountain soil. The failures of van Ermengem³ to isolate *B. botulinus* from various earth samples, such as garden soil, dirt from streets, canal slime, mud from ponds and rivers, etc., together with the relatively rare occurrence of botulismus

Received for publication, July 26, 1922.

* This study was aided by grants from the National Canners Association, the Canners' League of California and the California Olive Association.

¹ Jour. Bacteriol., 1919, 4, p. 541.

² Ztschr. f. Hyg. u. Infektionkr. 1897, 26, p. 482.

³ Handbuch d. pathog. Mikroorganismen, 1912, 4, p. 936.

in certain states of the Union, indicated that the anaerobe was probably not omnipresent but occurred only in certain soils, probably due to accidental contaminations from heavily infected areas or possibly manure or animal excreta. These and similar considerations prompted an extensive cultural study of the telluric distribution of *B. botulinus*. Without an adequate knowledge of the biochemical activities and growth requirements of the bacillus at the time these studies were begun, it was naturally impossible to foresee the scope of the investigation. When the problem was approached, the prevailing idea of the intestinal habitat of sporulating anaerobes prompted the examination of cultivated and manured garden, field and pasture soils. Considerable attention was paid to the distribution of *B. botulinus* in various olive groves and on vegetables and fruits bought in the open market of various cities and towns of the state. The examination of control specimens and with it the frequent demonstration of *B. botulinus* in uncontaminated mountain soil, however, changed the original point of view so that, in addition to sampling field specimens in the vicinity of rural communities in which human or animal botulism had occurred, soil from uninhabited, virgin territories was collected and cultures made. Simultaneously, numerous specimens of manure were studied, and the persistence of the spores of *B. botulinus* in the intestines of laboratory animals, as well as the factors responsible for the growth of the anaerobe in various earth specimens under laboratory conditions, was experimentally investigated. In order to verify some of the observations made in California, a broad investigation of soil and vegetable samples procured from every state of the Union, Canada, Hawaii, Alaska, China and Europe was subsequently made. This paper deals with the findings made on 607 specimens of soil and vegetables collected in California, and is mainly intended to serve as an introduction to the communications dealing with the distribution of the spores of *B. botulinus* in the United States.

COLLECTION AND STUDY OF SAMPLES

Soil specimens (200-500 gm. samples) were collected in small, carefully sterilized, sanitary tin containers (No. 1 cans). The lids of the containers were sealed and covered by sterile linen cloth. As a rule, samples of surface and subsurface soils (about 10 inches) were procured. Vegetables and fruits, as found in the open market or gardens, were packed in tight wooden boxes. Extreme precautions were taken to avoid extraneous contaminations in transit, as well as in the laboratory.

The writers are deeply indebted to Dr. J. C. Geiger, Epidemiologist of the U. S. Public Health Service, who carefully collected the majority of

specimens on which these studies are based. A few samples were obtained by the Inspectors of the Food and Drug Department, California State Board of Health, by collaborators, by assistants and by veterinarians, who reported cases of "forage poisoning."

Adequate samples, varying from 10 to 100 gm. of soil, vegetables, etc., were treated according to the procedures outlined in a former paper.⁴ All cultures were made with heated specimens in beef heart peptic digest liver broth. A number of the early cultures were incubated for from 4 to 6 weeks instead of 10 days, this shorter period was later adopted as a standard procedure. It is, therefore, not unlikely that on account of the deterioration of the toxin following the longer periods of incubation some of the specimens containing the spores of *B. botulinus* were not recognized. The cultures were always tested on guinea-pigs and the type of toxin determined on the same species of animal. About one-third of the toxic cultures produced characteristic symptoms of intoxication, but the nature of the toxin could not be ascertained by an antitoxin neutralization test. Sufficient experimental evidence has, however, been collected and presented in the first paper of this series, to support the conclusions that those soil or vegetable samples producing weakly toxic cultures contained only a few spores of *B. botulinus*. In a study dealing with the distribution of *B. botulinus* in nature, the findings pertaining to these weakly toxic specimens, even if they are not conclusively proved to be the result of *B. botulinus* by antitoxin tests, deserve recognition and are therefore included in the discussion.

EXPERIMENTAL DATA

The survey made in California during the years 1920 to 1922 covered the following phases of the problem: (1) A search for the spores in the soil or its products collected from gardens in widely separated localities in which human or animal botulism had occurred during the years 1919 to 1922; (2) systematic sampling of soil, vegetables and fruits from olive groves, spinach fields, which had furnished the raw materials, and later of products of packing plants, such as improperly sterilized canned products which had caused several outbreaks of human botulism; (3) culturing of fruits and vegetables bought in the open market; (4) toxicologic and cultural tests of feeds, hay, etc., supposed to have caused forage poisoning; (5) culturing of manure used in the fertilization of olive groves, vegetable gardens, etc.; (6) selective sampling and making of cultures of virgin soil collected in isolated areas of the Sierra Nevada, San Bernardino and Coast Range Mountains.

It is unnecessary to describe the various specimens and the time of the year during which the material was collected and cultures made. The results are summarized in table 1. For future reference, a presentation along geographic lines has been chosen and the findings previously reported by Dickson and Burke are included in the table. The survey is obviously not exhaustive, but the ubiquitous recognition of the spores in every section of the state made it unnecessary to extend the examination along broader telluric lines, although some of the early studies suggested a localization of the spores in certain districts and counties. This belief that the distribution of *B. botulinus* is confined to certain areas, towns, etc., followed the examination of a limited number of specimens and was readily disproved by the subsequent broader study of the problem. From a biologic point of view, intensive sampling of

⁴ Jour. Infect. Dis., 1922, 31, p. 501.

TABLE 1
RESULTS OF TESTS WITH SPECIMENS FROM VARIOUS COUNTIES IN CALIFORNIA

No.	Record	Location	Character of Specimen	Number of Samples	Number of Cultures	Total Number of Typed Cultures	Results			Remarks
							Untyped	Type A	Type B	Types A and B
1	182C	Alameda County Grizzly Peak, Berkeley Hills (1,759 ft.)	Virgin soil from new trail...	6	6	5	—	5	—	—
2	33B	Oakland.....	Hay (2) and chicken manure	3	3	—	—	—	—	—
3	15	Berkeley.....	One jar of spoiled string beans	1	2	2	—	2	—	—
4	Report	Berkeley.....	Three jars of string beans and gizzards of 2 chickens	5	5	5	—	—	5	Isolated by E. C. Dickson
5	4	Butte County Olive ranches, Oroville	Olives on trees, bird pecked and buried, hog manure	6	12	5	—	—	5	—
6	51	Packing plant, Oroville	Decayed Sevillano and dried Mission olives	3	3	—	1 (Sevillano)	—	—	—
7	81B	Packing plant, Oroville	Swelled cans of ripe pickled olives	3	3	1	—	—	1	Brine nontoxic
8	34	S. Ranch, Oroville.....	Soil (5), green olives and leaves (1)	6	15	—	—	—	—	Incubated for 12 weeks
9	35	O. Ranch, Oroville.....	Soil (6), green olives and leaves (1)	7	12	—	2 (soil)	—	—	Incubated for 12 weeks
10	36	P. Ranch, Oroville.....	Soil (7), green olives and leaves (2)	9	20	—	—	—	—	Incubated for 12 weeks
11	38	D. Ranch, Oroville.....	Soil (6), green olives and leaves (2)	8	18	—	—	—	—	Incubated for 12 weeks
12	39	E. Ranch, Oroville.....	Soil (6), manure (1), olives and leaves (2), fertilizer (1)	10	14	—	1 (soil)	—	—	One fertilizer positive; manure malignant edema
13	184	F. Ranch, Oroville.....	Soil, fertilized with manure	6	6	2	—	2	—	—
14	52-54 IV	Chico.....	Hay suspected of causing forage poisoning	4	4	—	—	—	—	Extract nontoxic
15	681	Chico.....	Hog manure.....	—	—	—	1	—	—	—
16	62B	Chico.....	Mill run and hog tackage...	2	4	—	4	—	—	—
17	67	Biggs.....	Soil.....	16	16	—	1 (crop)	—	—	Incubated for 28 days
18	73	Durham.....	Hay, alfalfa, maize, calf-meat, rice	8	8	—	1 (corn garden and burial ground)	—	—	—
19	100B	Packing plant, Oroville	Spoiled, pickled olives.....	9	9	—	1	—	—	—

20	10	Colusa County Colusa.....	Decomposed hay (2), moldy beanstalk and plant (2), soil (2)	6	10	1 (bean plant)	2 (soil)	1	—	—
21	20	Contra Costa County Pleasant Hill.....	Decomposed hay (forage poisoning)	—	—	—	—	—	—	Malignant edema
22	171	El Dorado County Lake Tahoe.....	Virgin soil.....	5	5	5	—	5	—	—
23	76	Fresno County King's River Canyon..	Spleen, intestine, liver, etc., of a horse	6	6	1 (intes- tines)	—	1	—	Forage poisoning (?)
24	76B	King's River Canyon..	Soil from pasture, hill top..	3	3	3	1	3	—	—
25	76A	Fresno hills.....	Soil.....	3	3	—	—	—	—	—
26	76B	Mountain valley.....	Virgin soil.....	3	3	1	—	1	—	—
27	180A	Telden Pass.....	Virgin soil.....	1	1	—	—	—	—	—
28	180	Glenn County Orland.....	Hay and feed.....	2	2	—	—	—	—	Incubated for 28 days
29	77	Humboldt County Eureka.....	Soil from vegetable gar- dens	8	8	—	—	—	—	—
30	77B	Eureka.....	Vegetables from open mar- ket	10	10	—	2 (pars- nips)	—	—	—
31	77B II	Eureka.....	Soil from vegetables.....	2	2	—	—	—	—	5 gm. sample
32	180A	Inyo County Plute Pass.....	Virgin soil.....	1	1	—	—	—	—	—
33	66B	Kern County Bakersfield.....	Straw and burned hay (for- age poisoning)	2	4	—	—	—	—	—
34	5	Los Angeles County Long Beach, La Mosa Ranch	Moldy olives (3), soil (3)....	6	6	—	—	—	—	—
35	24A	52d St., Los Angeles...	Garden soil, upper and lower layers	6	6	—	—	—	—	—
36	26	Whittier.....	Hay (1), soil (1).....	2	4	—	—	—	—	—
37	29A	Whittier.....	Manure of sick horses.....	2	2	—	—	—	—	—
38	54	Whittier.....	Hay (2), manure (2), corn (3)	7	7	4 (hay and manure)	—	2 (manure)	2 (hay)	—
39	74A	Whittier.....	Hay (1), manure (2), bar- ley (1)	4	4	—	1 (hay)	—	—	—
40	50	Los Angeles.....	Cadaver of chicken.....	1	11	1 (9)	—	1 (9)	—	See Human Reports 18 and 19
41	56	Los Angeles.....	Chicken feed.....	2	2	—	—	—	—	—
42	50	37th Ave., Los Angeles	Soil and string beans.....	2	2	—	—	—	—	—
43	58	Sylmar Ranch.....	Soil.....	3	6	1	—	1	—	—
44	58	Sylmar Ranch.....	Manure (2), Mission olives from trees	5	10	—	3 (olives, tree and manure)	—	—	—
45	58	Sylmar Ranch.....	Soil.....	5	10	2	4	2	—	—

TABLE 1—Continued
RESULTS OF TESTS WITH SPECIMENS FROM VARIOUS COUNTIES IN CALIFORNIA

No.	Record	Location	Character of Specimen	Number of Samples	Number of Cultures	Total Number of Typed Cultures	Results			Remarks	
							Untyped	Type A	Type B		Types A and B
46	58	Los Angeles Co.—Cont. Sylmar Ranch.....	Olives from trees, leaves, etc.	4	8	1	2	—	1	—	
47	129B	51st St., Los Angeles...	Surface soil, chicken droppings, goat manure	4	4	—	2 (soil)	—	—	—	See Chicken Outbreak 21
48	129	Laguna Ranch, Los Angeles	Feed, manure, stagnant water, sewage (5)	9	9	—	4 (manure and beet pulp)	—	—	—	Bovine botulism suspected
49	134	New Hall Ranch.....	Soil (6), manure (2), decayed meat (1)	9	9	—	—	—	—	—	Malignant edema
50	135	New Hall Ranch.....	Pasture soil.....	12	12	1	4	—	1	—	Five vibrios septique
51	139	Los Angeles.....	Barn feed and manure.....	2	2	—	—	—	—	—	
52	181	Los Angeles.....	Corn husks and potatoes...	3	5	1	—	1	—	—	
53	224	Glendale.....	Three chickens and home canned corn	4	1 (6)	1 (6)	—	1 (6)	—	—	See Chicken Outbreak 107; isolated
54	136	Pasadena.....	Sewer farms, soil and sewage	4	4	—	2 (soil)	—	—	—	
55	136	Mount Lowe (6,100 ft.)	Soil (5), trail dust (1).....	6	6	2	1	2	—	—	Trail dust contaminated with horse feces containing B. tetani
56	7	Marin County	Beanstalks (1), soil (2).....	3	5	—	—	—	—	—	See Human Report 15
57	8	San Rafael.....	Beanstalks and soil.....	2	4	—	—	—	—	—	
58	25	San Rafael.....	Soil.....	5	10	—	—	—	—	—	
59	K2	San Rafael.....	Specimens of vegetables and fruits (cherries, beans, beet root, asparagus, carrots)	12	25	3	3 (beans, vine, asparagus)	2 (asparagus)	1 (asparagus)	—	
60	181A	San Rafael.....	Two fresh clams.....	2	2	2	—	2	—	—	
61	202	Mt. Tamalpais.....	Virgin soil from Dipsea trail, Lone Pine, Forest Deer Park and Alpine trail	8	8	5	—	2 Lone Pine and Alpine trail	—	2 Forest Deer Park and Dipsea	
62	52B	Mariposa County Yosemite Valley.....	Virgin soil, Camp C-6, El Capitan, Meadow and Ranger Station on Bridal Veil	4	8	2	—	1 Forest Deer Park 2 (Camp 6)	—	—	
63	122	Yosemite Valley.....	Rockslide virgin soil, 1,000 feet above valley	4	8	3	1	3	—	—	
64	122	Yosemite Valley.....	Excavation in virgin soil 1 foot deep	6	6	6	—	6	—	—	
65	171	Gentry.....	Virgin soil.....	8	8	2	—	1	1	—	

66	171	Mono County Mono Lake.....	Virgin soil.....	6	6	2	1	2	1	—
67	42	Napa County Napa.....	Garden soil and vegetables.	13	13	—	—	—	—	—
68	47	Calistoga.....	Garden soil, vegetables, hog and horse manure	10	10	—	—	—	—	—
69	114	Placer County Colfax.....	Soil from horse corral.....	2	2	—	—	—	—	—
70	79B	Roseville.....	Garden soil (2), pasture soil (2)	4	4	—	—	—	—	—
71	172B	Plumas County Feather River Canyon Riverside.....	Corral dirt and water (for- age poisoning)	4	4	—	—	—	—	—
72	183	Sacramento County Sacramento.....	Virgin soil.....	12	12	7	—	6	—	1
73	135	Sacramento County Sacramento.....	Soil from orange groves, garden 1, Mt. Rubidoux	5	5	—	—	—	—	—
*	Report	Sacramento County Sacramento.....	Jar of string beans, locally grown	—	—	—	—	—	1	—
*	Report	San Benito County Hollister.....	Water flies, slugs, sowbugs, earthworms, chicken ma- nure, straw, dirt, mud, hog manure	—	—	1	—	—	1 (hog manure)	—
74	135B	San Bernardino County Cucumbo.....	Soil from gopher holes.....	2	2	—	—	—	—	—
75	136	Sewer farms.....	Soil, top and deep.....	6	6	2	2	—	2	—
76	55	San Diego County San Diego.....	Soil, weeds and goat ma- nure	6	8	4	—	4 (top soil and weeds)	—	—
77	58	Escondido.....	Soil (3), potatoes (1), beans (1), hog manure (1)	6	13	2	—	2 (bean garden soil and hog manure)	—	—
78	28	San Francisco County Golden Gate Park....	Fertilized soil, deep and surface	5	10	—	—	—	—	—
79	32	Golden Gate Park....	Fertilized soil from nursery	5	10	—	—	—	—	—
80	62C	San Joaquin County Stockton.....	Damp hay.....	1	3	—	—	—	—	—
81	46	San Mateo County Menlo Park.....	Cadavers of chickens and soil	5	10	1	—	1	—	—
82	49	Menlo Park.....	Soil (8), bean leaves (1)....	9	9	—	—	—	—	—

Malignant edema; garden
soil

1918, Mrs. G. S. Burke

1918, Mrs. G. S. Burke

Spinach responsible for
outbreak 63 grown in
these farms
Suspected botulism among
goats

See Chicken Report 53

Incubated for 10 weeks

TABLE 1—Continued
RESULTS OF TESTS WITH SPECIMENS FROM VARIOUS COUNTIES IN CALIFORNIA

No.	Record	Location	Character of Specimen	Number of Samples	Number of Cultures	Total Number of Typed Cultures	Results			Remarks
							Untyped	Type A	Type B	
83	9	Santa Barbara County	Straw (forage poisoning)...	1	2	1	—	—	1	Isolated by E. D. Dickson, see Report 1 Isolated by Mrs. G. S. Burke
84	24	Santa Maria.....	Hay (1), soil (3).....	4	4	3	—	—	3 (soil and hay) 1 (foliage)	
85	127	Monterey.....	Peas, foliage and soil (2)...	4	4	3	—	2 (soil)	—	
86	127B	Santa Barbara.....	Virgin soil and olives (San Carlo Ranch)	3	5	5	—	5 (olives, leaves and soil)	—	
87	202B	Monterey.....	Soil from Bishop Ranch....	1	1	1	—	—	1	
88	1	Santa Clara County	Chicken cadaver.....	1	10	1	—	1 (intestines)	—	
89	2	Saratoga.....	Garden soil, string beans....	2	12	2	—	2 (soil)	—	
90	40	Saratoga.....	Soil (7), fruits (4), vegetables (6), leaves (2), sewage	20	27	1	—	1 (soil, orchard)	—	
91	31	Morgan Hill.....	Fruits and vegetables.....	5	8	1	1 (soil)	1 (apricot)	—	
92	43	Los Gatos.....	Soil (4), plums (2), bean leaves	7	9	—	4 (soil)	—	—	
93	61	San Jose.....	Vegetables from open market	15	15	5	2	5 (beans, celery, potato, turnips, lima beans)	—	
*	Report	San Jose.....	Chicken cadaver.....	—	—	—	—	1	—	
*	Report	Palo Alto.....	Peas, beans, peaches, caterpillar, corn, cherries	—	27	2	—	2 (cherries)	—	
94	57	Santa Cruz County	Garden soil and bean leaves	5	5	—	—	—	—	
95	70B	Boulder Creek.....	Virgin soil.....	6	6	—	1	—	—	
96	219	Sonoma County	Spoiled string beans, locally grown	1	1	1	—	1	—	
97	113B	Stanislaus County	Garden soil.....	6	6	6	—	6	1 (spotted bean leaf)	
*	Report	Newman.....	Spotted leaves, pods, bush string beans, ants, spiders, bugs, grass hoppers	—	23	1	1 (spider, etc.)	—	—	
*	Report	Oakdale.....	Moldy hay.....	1	1	1	—	1	—	
									1918, Mrs. G. S. Burke	

98	64	Tehama County D. Ranch near Corn- ing	Soil (4), bird pecked ripe olives	6	13	—	—	—	—	—
99	51	Tulare County Lindsay	Sh. Ranch soil (7), green olives (5), leaves (1)	13	13	1	1	—	—	—
100	51	Lindsay	G. Ranch soil (2), green olives (4)	4	4	1	—	—	1 (soil)	—
101	51	Lindsay	Dr. Bruce soil (2), green olives (4)	6	6	—	—	—	—	—
102	51	Lindsay	I. Ranch, olives and leaves...	2	2	2	—	—	—	2 (leaves and green olives)
103	51	Lindsay	Eck. Ranch soil (2), olives (2)	4	4	—	2 (soil) and olive	—	—	—
104	51	Lindsay	Els. Ranch soil (1), olives (2), manure (2)	5	5	—	—	—	—	—
105	51	Lindsay	Orange grove soil...	2	2	—	—	—	—	—
106	51	Packing plant, Lind- say	Scrapings, plucking barrels (2), shipping barrels (1), olive dumps (1), convey- ors (2), discarded olives (7)	13	13	1	1 (dis- carded olives)	1 (scrap- ings ship- ping bar- rel)	—	—
107	171	Tuolumne County Tioga Pass	Virgin soil...	6	6	2	—	2	—	—
108	41	Ventura County Oxnard	Soil (1), hay (1), horse ma- nure (1)	3	6	—	—	—	—	—
109	80	Yolo County Davis	Horse (2) and hog (2) feed, manure (4)	8	22	—	—	—	—	—
110	87	Davis	Horse, hog and cow feed, manure	8	8	—	—	—	—	—
111	22	Unassigned—General Distribution	Vegetables (2), fruits (1)...	4	10	2	—	—	—	Toxin deteriorated before typing
112	1K	San Francisco	Fruits (11)...	11	16	3	3 (tur- nip tops, plum(s) peas)	2 (tur- nips, cher- ries)	1 (peaches)	—
113	27	San Francisco	Vegetables...	6	14	2	—	—	2 (beet(s))	—
114	30 and 33	San Francisco	Spinach, string beans, etc.	11	11	—	—	—	—	Incubated for 6 weeks
115	49B	San Francisco	Beet tops, corn, potatoes...	4	6	2	1 (corn)	—	2 (beet tops)	—

certain mountain areas deserves attention in the near future, but from a practical standpoint, at least, it is unnecessary to devote material and energy to an additional study of specimens collected in California. It has been definitely proved that the spores are widely distributed and may be found in soils and their products from any district or locality.

An analysis of the data presented in table 1 shows that 624 specimens have been examined by means of 894 cultures. These figures include also the samples of spoiled home canned products, tissues, etc., procured from animals after necropsies had been performed. Six hundred and seven specimens, representing samples of soil, vegetables, feeds, fruits and manure, were selected for further consideration and discussion; 179, or 29.4% of the specimens produced toxic cultures; 89 contained type A; 26 type B, and 4 type A and B *B. botulinus* toxins, while 60, or 9.8% of the cultures were not sufficiently toxic to furnish conclusive antitoxin neutralization tests. One hundred and nineteen, or 67.6% of the 179 toxic cultures were properly identified. Nearly three-fourths, or 74.8% of the typed cultures contained type A, showing clearly the predominance of this resistant type in the soil of California. Type B was found in 22.1% and a mixture of types A and B in 3.1% of the samples. The latter findings are important, as they definitely indicate that the 2 types of *B. botulinus* may coexist in the soil. The real biologic significance of the tabulated data was, however, not realized until the cultural findings were classified according to their origin. This classification is presented in chart 1.

For the sake of clearness, it is considered advisable to discuss the various columns separately.

1. *Virgin Soil Specimens*.—Only samples of soil collected from areas definitely known to be free from contaminations of animal excreta are included in this group; in fact, the majority of specimens represent earth of the Sierra Nevada or Coast Range Mountains. The first positive proof that *B. botulinus* exists in virgin soil was furnished by a number of soil specimens removed from a rockslide which occurred March 1, 1921, about 1,000 feet above the floor of the Yosemite Valley (El Capitan side, Big Oak flat). Samples were taken on April 20. Repeated cultural tests revealed the existence of spores of *B. botulinus*; the organism being isolated in pure culture on numerous occasions. Identical findings were made with soil collected from an excavation 4 feet deep in the heart of the valley, in which pollution with manure, etc., was absolutely excluded. These findings have subsequently been strengthened by the examination of soil specimens from the Sierra Nevada ridge near Tioga Pass, the Mono Lake and Lake Tahoe region, Feather River Canyon, etc. A total of 78, with 45, or 57.6%, toxic cultures has been studied. Forty-three samples of the latter cultures have been identified, and the predominance of type A has been definitely established. One sample, which originated from the vicinity of Gentry, Sierra foothills, Yosemite National Park, contained *B. botulinus* type B, while 2 specimens from Mount Tamalpais, near San Francisco, and 1 from the mouth of the Feather River Canyon contained mixtures of types A and B. Moreover, it is evident from the ease with which *B. botulinus* was isolated from the soil enrichment cultures and from the relatively low percentage of weakly toxic cultures that the spores are quite numerous in the virgin soil specimens. The evidence strongly supports the contention that *B. botulinus* is a common member of the soil flora in the Sierra Nevada and Coast Range Mountains.

2. *Garden, Orchard and Cultivated Field Soil Specimens*.—The samples included in this column originated from soil areas which were known to have

been fertilized either quite recently, or at least in the course of the last two to three years. In some instances, commercial fertilizer, but usually barnyard manure, had been employed. The majority of specimens were collected in olive groves and vegetable gardens. Recently a number of farms growing spinach and regularly flooded with sewage from Pasadena and other places have

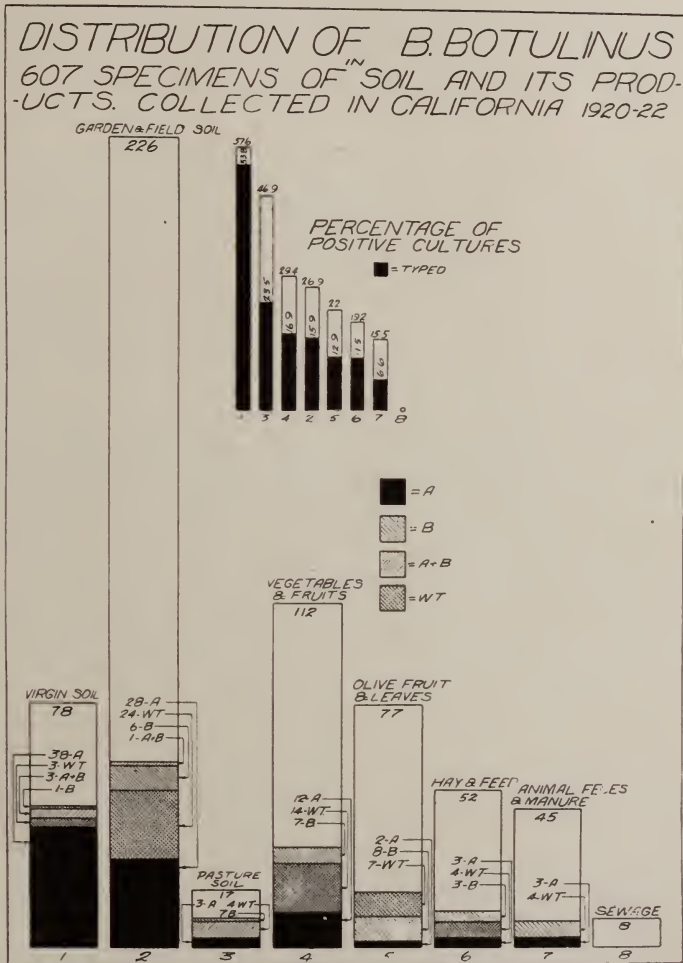


Chart 1

been tested. Fifty-nine cultures, or 26.9%, of the total 226 specimens were toxic. A relatively high percentage (40.6%) of the cultures was weakly toxic and could not be identified by antitoxin tests. These results are due to one or two conditions: either the number of spores of *B. botulinus* per gram of soil are few, or the concomitant anaerobes outgrow the toxin producers in the enrich-

ment cultures. Type A was the predominant type, although type B appeared more frequently than in the virgin soil samples. The proportion of type A to type B for the virgin soil specimens was 38:1, while it shifted to 4.66:1 for the garden and cultivated field soil samples. The ranches from which the samples were taken are located in the Sacramento or San Joaquin Valley and receive their irrigation water from the Sierra Nevada Mountains. It is not unlikely that the fields are seeded with the type A spores from the water sources which are heavily infected with this organism. What happens to these spores can only be discussed after the various other columns have been carefully analyzed. It must, however, be concluded that the spores of *B. botulinus* are less frequently encountered in garden or cultivated field soil than in virgin mountain soil.

3. *Soil of Pastures*.—A small number of soils collected from pastures have been studied for the presence of *B. botulinus*. The specimens were collected from places on which horses or cattle had died from diseases diagnosed by local veterinarians as "forage poisoning." In every instance, the soil was markedly polluted with animal excreta, and it is significant that 9 of the 17 specimens produced "malignant edema" in guinea-pigs. Three of the 17 cultures contained type A, while one was neutralized by a type B antitoxin; 4 cultures could not be identified. Unfortunately, control tests of pasture soils not contaminated with animal manure were not made, and the limited number of specimens does not permit any definite conclusions.

4. *Vegetables and Fruits*.—This group of cultures was prepared with specimens either bought in the open market of San Francisco, San Jose (Santa Clara Valley), Los Angeles, Eureka (Humboldt County), or collected in gardens which had supplied the vegetables responsible as canned goods for cases of human or animal botulism. Cultures have been taken from potatoes, beets, asparagus, etc., heavily covered with soil, or fruits picked from the trees slightly contaminated with dust. In this connection it was noted that for example bird pecked cherries or bruised, partially spoiled apricots gave positive cultures more frequently than sound fruit; on one occasion, the soft, moldy blemish of an apricot gave a toxic culture, while the sound skin furnished a nontoxic culture. It is not unlikely that the spores of *B. botulinus* in the dust of the soil are fixed by the sticky sap of the bruised fruit. Thirty-three, or 29.4%, of the 122 specimens of vegetables and fruits produced toxic cultures. Fourteen, or 42.4%, of the cultures were weakly toxic and non-neutralizable, while 12 were neutralized by type A and 7 by type B antitoxins. The findings on the vegetables and fruits correspond in a general way with those made on the soil specimens collected in gardens and cultivated fields. The relatively high percentage of type B toxins has been repeatedly confirmed by the isolation of *B. botulinus* type B from the enrichment cultures. The vegetables, such as beets, asparagus, etc., bought in San Francisco were grown in truck gardens heavily fertilized with hog manure. Specimens of the fertilizer were tested for spores of *B. botulinus* with negative results. Soil products procured from plots recently planted with vegetables furnished enrichment cultures which contained type A toxins. Invariably the repeated examination of the soil demonstrated the same type. So far as the limited material permits of deductions, it is evident that the products of a soil which has been manured and cropped for many years more frequently contains the spores of *B. botulinus* type B than those of type A. Fruits and vegetables are excellently suited to demonstrate this distribution, probably on account of the relative minority of other proteolytic anaerobes which ordinarily destroy the toxins or overgrow *B. botulinus* present in specimens prepared from manured or fertilized soil.

5. *Olives, Foliage from Trees, Specimens from Olive Packing Plants.*—As a number of tragic human botulism outbreaks due to pickled ripe olives instigated the investigation on the distribution of *B. botulinus* in nature, it became necessary to follow the path of this organism from the olive grove to the packing plant. From the data summarized in column 5, it is evident that *B. botulinus* was readily demonstrated on olives. Not only were the leaves and fruits on the trees found to be contaminated, but the scrapings from the shipping barrels transporting the ripe olives from the groves to the cannery, as well as the refuse dumps composed of spoiled, discarded olives, supplied toxic cultures. Furthermore, several tins of ripe pickled olives, processed at low temperatures and unprocessed, so-called "bulk olives," gave pure cultures of *B. botulinus* type A. The frequent finding of type B spores only on the foliage and green fruits is closely connected with the predominance of this type in the manured soil of the ranches. This type is probably not very resistant against physical and chemical agents, rarely survives the pickling and preserving processes and has, therefore, not been found in the packed products.

Seventeen, or 22.0%, of the 77 olive specimens produced toxic cultures; the proportion of type A to type B was 1:4. The foliage and green olives collected from the trees furnished 10% weakly toxic enrichment cultures. From a practical standpoint, it is beyond question that *B. botulinus* is frequently carried on the ripe fruits into the shipping barrels, pickling vats and the packing plant. The question: Why did the organism under these prevailing conditions produce its toxin only in a very few nonsterile glass containers (probably 9 to 10 containers) is certainly not solved by the foregoing findings. It is not our purpose to enter into a theoretical discussion of a problem which was unfortunately not investigated when the conditions in the packing plants offered excellent opportunities for interesting disclosures. It is not unlikely that during and shortly after the war, the collection of ripe olives in the orchards was not conducted with a great deal of care. The raw product, heavily contaminated with soil, underwent further deterioration and the resistant spores were permitted to increase by prolonged storage in shipping barrels and pickling vats. The common practice of preserving the olives by heat not exceeding 212 F. produced in some containers an environment suitable for the growth of *B. botulinus* and its toxin. It is, however, now fully recognized that efficient sterilization of fresh and carefully handled ripe olives will prevent further outbreaks of botulism.

6. *Hay, Straw and Animal Feeds.*—One-half of the specimens and cultures from which this column is computed originated from outbreaks of "forage poisoning," while the other half consisted of similar material collected in the vicinity of the outbreaks. Watery or saline extracts were usually found to be nontoxic for guinea-pigs, although 10, or 19.2%, of the 52 samples produced toxic cultures. Type A, as well as type B *B. botulinus* were demonstrated and isolated from the cultures. The percentage of positive cultures and the relative proportion of the two types of *B. botulinus* differed in no way from that established for the soil supplying the hay or feed. Furthermore, no differences were noted between the feeds suspected of having caused forage poisoning and those procured from sound haystacks or feeding troughs. The assumption that the finding of *B. botulinus* in the feed proves the botulism nature of "forage poisoning" is not supported by the foregoing observations. In fact, it is shown that the mere cultural demonstration or isolation of *B. botulinus* in feeds, vegetables, etc., cannot be considered diagnostic of botulism.

7. *Manure and Fertilizers.*—Three, or 6.6%, of the 45 specimens contained the spores of *B. botulinus* type A. Four samples furnished weakly toxic enrich-

ment cultures. As a rule, large specimens varying from 100 to 500 gm. were employed. The low percentage of positive cultures is either due to an absence of the spores or the methods employed are unsuitable to detect the organism when present in relatively small numbers. It is not intended to discuss here the importance of some of the herbivorous animals as carriers or disseminators of *B. botulinus* spores, but the evidence strongly suggests that manure or fertilizers contribute relatively little to the pollution of the soil with this bacillus. On numerous occasions, parallel examinations of ranch soil and its fertilizing material revealed the presence of *B. botulinus* in the former and its absence in the latter. Moreover, it has been repeatedly pointed out that earth contaminated with animal feces is rarely infected, while mountain and forest soil in California are practically always infected with *B. botulinus* spores. In this respect the organism differs from its related species the tetanus bacillus. (See 54, Table 21.) It is not unlikely that occasionally the spores ingested with the feed are eliminated in the feces and in this manner herbivorous animals may act as distributors of the spores. Experimental studies to be presented by E. J. Easton in another paper confirm this conclusion and amplify the data outlined in this paragraph.

8. *Sewage*.—An extensive outbreak of botulism in chickens was investigated from both a pathologic and a bacteriologic standpoint. Vegetable and soil cultures revealed the fact that the garden in which the beans responsible for the fatalities were grown had been regularly fertilized by means of sewage and contained the spores of *B. botulinus*, type A. It was later found, however, that certain truck gardens furnishing spinach to canneries were constantly treated with sewage. Furthermore, a number of specimens of heated and unheated sewage were cultivated with entirely negative results, so that the evidence deduced from this outbreak among chickens can hardly be considered strikingly opposed to the previously established conception of the telluric origin of *B. botulinus*. It has been demonstrated in this laboratory that human excreta produce, as a rule, nontoxic enrichment cultures, irrespective of the fact that spores are frequently ingested on raw vegetables and fruits. Evidence is still lacking to prove that multiplication takes place in the human intestinal canal or in sewage and that the latter contributes to the contamination and pollution of the soil in certain districts or areas of this state.

In the light of the findings discussed under 7 and 8, it is evident that in California at least *B. botulinus* is not disseminated by animal excreta nor is the intestinal canal the natural habitat of this organism. This conclusion will be supported by data presented in subsequent papers, but it appears of interest to investigate briefly the distribution of the two types of *B. botulinus* as determined by this study. The predominance of type A in virgin soil and the occasional or relatively frequent occurrence of type B in manured gardens or ranch soil is sufficiently constant to suggest the existence of a biologic phenomenon. Data to be mentioned in a paper dealing with the morphologic and biochemical activities of 100 strains of *B. botulinus* indicate that the B types more often show variations in their cultural and serologic characteristics than the A types. In brief, it is believed that the former types are mutants of the latter. The biologic, physical and chemical

influences encountered by the stable or fixed type A in cultivated and manured soil are probably responsible for the creation of a new adaptation type which can develop more readily in the new environment and as such preserve the genus *Botulinus*. The mutation is in this case favorable to the existence of the organism and enables it to multiply actively in an environment which is less suitable than the virgin, untilled soil. This interpretation of the origin of type B is quite in accordance with the observation that proteolytic anaerobes mutate more frequently than nonproteolytic types. One recalls in this connection the recently published paper of H. H. Heller⁵ on mutations in the genus *B. tetani* and some of the studies of Löhnis⁶ dealing with the life cycles of soil bacteria. It is obvious that this explanation deserves careful experimental investigation and is herewith offered as a working hypothesis for a field which has not only biologic but also some practical importance.

CONCLUSIONS

The examination of 624 specimens of soil, vegetables, fruits, feeds, manure and sewage collected in 36 counties of California and studied by means of 894 cultures definitely indicates that the spores of *B. botulinus* are very widely distributed. Approximately 30% of the samples produced toxic cultures. 74.8% of the identified toxins were neutralized by a type A, 22.1% by a type B and 3.1% by a polyvalent antitoxin.

The evidence strongly suggests that the natural habitat of *B. botulinus* is found in virgin mountain or forest soil. *B. botulinus* is also present in cultivated garden and field soils and their products. Vegetables and fruits bought in various cities and towns of California carry the spores of *B. botulinus*.

B. botulinus type B occurs predominantly in cultivated and manured soils and is probably a mutant of the fixed type A.

⁵ Jour. Infect. Dis., 1922, 30, p. 33.

⁶ Jour. Agric. Res., 1916, 6, p. 675.

THE DISTRIBUTION OF SPORES OF *B. BOTULINUS* IN THE SOIL OF A RESTRICTED AREA IN CALIFORNIA. III *

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Santa Barbara, California

Since the fatal outbreak of poisoning in 1919 in various parts of the United States due to the consumption of California olives which had been contaminated by *B. botulinus*, a widespread interest has been aroused, particularly in this state, as to the distribution of the spores of this bacillus. Aside from their desire to suppress this menace to the public health, various organizations, both national and local, financially interested in the growing and packing of fruits and vegetables, have greatly aided by grants of money the universities and public health laboratories in an intensive study of the different phases of this important problem. Burke¹ had found spores of *B. botulinus* on fruit and vegetables and the leaves of trees and plants in widely separated sections of California. It has been my privilege to read the manuscript of a monumental work on the distribution of the spores of *B. botulinus* in nature, by Meyer and Dubovsky, a portion of which appears in the form of several papers in the present issue of this journal. In one of these papers,² soils and other samples from many different localities in California, from the higher mountain regions, through the pasture lands of the foothills to the cultivated valleys, were studied, but in no case have many samples of soil been taken from several places within a comparatively restricted area. It is not within the scope of this paper to enter into a discussion of the many interesting features suggested by the work of these authors that such a study would present. The factors involved in the distribution of the two types of *B. botulinus* in soils, their origin, telluric differences and analogy to the distribution of *B. tetani* and other soil anaerobes are presented. The authors believe it possible that type A has its origin in the virgin soils of the mountain ranges, is swept down with such soils into the valleys, and that perhaps type B is merely a mutant of type A. The following report of the results

Received for publication, July 26, 1922.

* This paper confirms and enhances the conclusions drawn in Paper II. It is a great pleasure to include it in the series of publications dealing with the distribution of *B. botulinus* in nature. K. F. Meyer.

¹ Jour. Bacteriol., 1919, 4, p. 5.

² The Distribution of the Spores of *B. botulinus* in California, this issue.

of some tests of soils for the presence of *B. botulinus*, which I undertook in the summer of 1921, may be of some interest in connection with their work.

In preparing for this study, some time was spent at the laboratory of the George Williams Hooper Foundation for Medical Research, University of California, where every facility was placed at my disposal for acquiring the technic employed by the workers in their investigations. In order that my results might be comparable to those

TABLE 1
RESULTS OF AUTHOR'S EXPERIMENTS

1. S. walnut orchard, Carpinteria.....	10 miles east of Santa Barbara	<i>B. botulinus</i> , type A
2. F. lemon orchard, Summerland.....	8 miles east of Santa Barbara	<i>B. botulinus</i> , type A
3. G. olive orchard, Summerland.....	7 miles east of Santa Barbara	<i>B. botulinus</i> , type A
4. A. lemon orchard (twice), Montecito.....	6 miles east of Santa Barbara	<i>B. botulinus</i> , type A
5. C. vegetable garden, Montecito.....	5 miles east of Santa Barbara	<i>B. botulinus</i> , type A
6. Chicken droppings uncontaminated by soil (enclosure within above vegetable garden); 3 samples at different times	5 miles east of Santa Barbara	<i>B. botulinus</i> , weak toxin
7. Mixture fresh horse manure from 3 stalls, Bluebird Stables, Montecito	4 miles east of Santa Barbara	Negative
8. MacK. garden	City of Santa Barbara	<i>B. botulinus</i> , type A
9. Mixture wheat, cornmeal, rolled oats, sunflower seed, Union Commission Co.	City of Santa Barbara	Negative (<i>B. welchii</i> infection)
10. Walnut orchard, Goleta.....	6 miles west of Santa Barbara	Negative
11. B. Ranch, bean field.....	8 miles west of Santa Barbara	<i>B. botulinus</i> , type A
12. Summit Santa Ynez Mountains.....	About 15 miles east Refugio Pass; elevation about 3,500 feet; sample taken (away from any trail) in thick underbrush	<i>B. botulinus</i> , type A

obtained in that laboratory, the technic thus acquired was adhered to and is fully described by Dubovsky and Meyer³ in the first paper of their series.

The work described in this paper embraces a study of a few soils taken from a narrow strip of coast line about 18 miles long in Santa Barbara County, Calif., from Carpinteria on the east, through the city of Santa Barbara to the Bishop Ranch on the west. This strip averages about a mile and a quarter in width and is backed by the abruptly rising mountains of the Santa Ynez Range. The soils varied from light, sandy and rich loam to heavy clay.

³ An Experimental Study of the Methods Available in the Enrichment, Demonstration and Isolation of *B. botulinus*, this issue.

Some preliminary studies were first undertaken with 3 strains of *B. botulinus* and a soil sample from the Yosemite Valley sent me by Dr. K. F. Meyer. I was able to confirm his findings for this soil (*B. botulinus*, type A isolated) as well as for a soil sample taken by him personally from an orchard near Santa Barbara. Each of my samples was made up of a mixture of soil from the surface to a depth of about 6 or 8 inches, often from a gopher mound, and was taken with the usual aseptic precautions and placed in containers sterilized at 170 for 2 hours.

My results are given in table 1.

It is to be noted that the toxin from the cultures of chicken droppings, though giving definite symptoms of *B. botulinus* poisoning in experimental animals, was too weak to type with antitoxin neutralization tests. Evidence has been given³ that in such cases few spores exist in the material examined.

Cultures or tests were made of most of these soils several times, as severe tetanus symptoms often developed in the inoculated animals before the *B. botulinus* symptoms. In some cases, a protective dose of antitetanic serum was also given.

During the latter part of these experiments, liver was added to the beef heart peptic digest broth mediums, which gave a stronger toxin of *B. botulinus*, thus largely obviating the difficulty with the tetanus toxin.

It has been shown by this investigation that the cultivated soils of a narrow strip of coast line in Santa Barbara County, Calif., are heavily contaminated with the spores of *B. botulinus*, type A, as well as with those of *B. tetani* and that the virgin soil from the mountain range behind this strip of land also contains the spores of *B. botulinus*, type A.

THE DISTRIBUTION OF THE SPORES OF *B. BOTULINUS* IN THE UNITED STATES. IV*

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It has been customary to consider California an endemic focus of botulism and the prevention of this intoxication a problem confined to the Pacific Coast States. Such a view is generally held in the East and Middle West, and statements have from time to time been issued which tend to absolve the territories east of the Rocky mountains and place the odium of botulism squarely on California. In a recent paper¹ it has been shown that this apparent prevalence of botulism in the last named state is in all probability due to the interest this disease has received at the hands of a number of workers, as a result of which data have been unearthed and analyzed which have thus far not focused attention in the other states of the Union. Furthermore, an unbiased observer will have noted that botulism is an acquisition of recent years and is closely associated with the use of home or commercially canned food products. During the war, economic reasons prompted the adoption of home canning procedures, while later prosperity on one side and domestic labor difficulties on the other led to the extensive consumption of commercially preserved, "ready to serve" foods of plant and animal origin. Since 1912, eighty-seven outbreaks of human and animal botulism have been caused by food products grown and packed in 20 states, exclusive of California. In this connection the following states and outbreaks should be mentioned: Colorado (3 human and 3 fowl), District of Columbia (1 fowl), Florida (1 human), Idaho (3 human), Illinois (1 human, 1 fowl and 1 horse), Indiana (1 human and 1 fowl), Iowa (1 human), Kentucky (1 mule), Main (1 human), Massachusetts (3 human), New Jersey (1 human and fowl), New York (4 human), Nevada (5 fowl), Ohio (2 human), Oregon (4 human and 2 fowl), Pennsylvania (1 human), Texas (1 human), Washington (14 human and 23 chicken), Wisconsin (1 human) and Wyoming (1 human).

Received for publication, July 26, 1922.

* This study was aided by grants from the National Cannery Association, the Cannery League of California and the California Olive Association.

¹ The Epidemiology of Botulism, to be published in a Bulletin of the U. S. Public Health Service.

This summary permits one conclusion: The spores of *B. botulinus* must be widely distributed in the United States. In order to confirm this, however, an examination of soil, vegetable and manure samples procured in every state in the Union was undertaken. It is proposed in this paper to summarize these studies. It will be shown that no territory within the boundaries of the United States can justifiably claim immunity from *B. botulinus*. Absence of human or animal botulism from a number of states cannot be attributed to the non-existence of the spores of the causative organism in the soil, but is probably the result of certain other factors, which will be discussed in the analysis of the 1,538 specimens secured for this investigation.

ORGANIZATION FOR COLLECTING AND CULTURE OF THE SAMPLES

The same principles as outlined in the preceding paper dealing with the collection of field specimens in California have been followed in this study. It is self-evident that the cooperation of a number of agencies had to be enlisted in order to make a complete and comparative survey. Dr. H. M. Loomis, Director of Inspection of the National Cannery Association, through his field staff in Ohio, Michigan, Illinois, Colorado, Wisconsin, etc., obtained and forwarded valuable samples from the cultivated fields supplying the raw material to important canneries in these states. Dr. C. M. Haring, Director, Agricultural Experiment Station, University of California, solicited and secured the cooperation of a large number of agricultural experiment stations. Sets of at least 20 to 30 specimens were procured through these agencies, namely, one earth sample each from a corral which had been used for cattle during the past year, a pig pen, a poultry yard, a field used for corn, for grain, for clover or as pasture during the past year, several samples of cornstalks, of moldy straw, of decaying vegetation, of bean pods and stalks, of peas, of fresh greens, such as beet tops, spinach, etc., and of roots, such as carrots, beets, etc. These specimens proved valuable from a comparative standpoint and furnished a number of important facts. The study dealing with the distribution of *B. botulinus* in the state of Washington was based on specimens collected by Dr. J. C. Geiger during his survey on botulismus made in the spring of 1921 at Yakima, Tieton, Toppenish and Yelm. State boards of health, universities and colleges from neighboring states—in particular, Nevada, Utah, Oregon, Arizona and Colorado—frequently submitted samples for examination and rendered valuable assistance in this respect. In order to verify the conclusions reached for California that *B. botulinus* is an organism of the virgin soil, Dr. J. C. Geiger collected, in September and October, 1921, from 25 to 50 earth samples each from the Glacier and Mt. Rainier National Parks, from the Ozark Mountains in Arkansas, from the foothills near Alexandria in Louisiana, from the foothills near Bath in Maine, from the Blue Ridge Mountains near Washington, D. C., from Mount Baker near Saranac Lake in New York and from the Allegheny Mountains in West Virginia.

It is not only a duty, but a great pleasure, to acknowledge the assistance rendered by the various laboratories, experiment stations, universities and individual staff members who devoted time and energy to the collection of specimens and, through their efforts, enabled the Foundation to make a fairly complete and comparative survey.

The samples were collected in containers which had been sterilized by dry heat at a temperature of not less than 170 C. for 2 hours, or by live steam at a pressure of at least 15 pounds for 30 minutes. When received in the laboratory, extreme precautions were taken to avoid mixture or contamination. Mass cultures were prepared according to the procedures outlined in the first paper of this series; in fact, all cultures were made with heated specimens in beef heart peptic digest liver broth and incubated for 10 days. Crude and unfiltered cultures were tested on guinea-pigs either by subcutaneous inoculation or feeding. The nature and type of the toxin were identified on the same species of animal. In a number of instances, pure cultures were isolated from the enrichment cultures.

EXPERIMENTAL DATA

The character of the specimens, the cultural results and the epidemiologic facts thus far determined are reported separately for each state. A geographic, alphabetical presentation has again been chosen to facilitate subsequent reference in the discussion.

Alabama.—In August, 1921, Professor Wright A. Gardner, Alabama Polytechnic Institute, Auburn, Alabama, forwarded 16 specimens: soil from hog pen (2), cow lot (2), oat field (1), clover patch (1), chicken yard (1), corn field (1), vegetable garden (2), onions (1), green corn stalks (1), old corn stalks (1), moldy hay (1), lima beans (1), and decaying vegetables (1). *B. botulinus* type A was found in the culture prepared with the green corn stalks.

Summary: 10 cultivated soils or animal corrals, 6 vegetables and feeds. Total: 16 with 1, or 6.2%, positive culture; 1 type A.

Arizona.—One human outbreak at Florence, commercially canned beets grown and packed in Ohio.

Miss Jane Rider, Director, State Laboratory, University of Arizona, Tucson, submitted, in February, 1921, one sample of "filaree" hay collected in the southern part of the state and suspected of causing forage poisoning in horses. The culture proved nontoxic.

Arkansas.—In May, 1921, Dr. William L. Bleecker, Bacteriologist, University of Arkansas, College of Agriculture, Fayetteville, furnished 21 specimens: soil from pasture (1), cow lot (1), clover field (1), grain field (1), corn field (1), hog lot (2), poultry yard (1), cow peas (1), radishes (2), spinach (1), soja beans (1), lettuce (1), cornstalk (1), corn fodder (1), rotted straw (1), decaying leaves (2), moldy straw (1), and moldy silage (1).

The rotted straw gave a culture containing the toxin of *B. botulinus* type B, while the soja beans, decayed leaves and the soil from the hog pen produced toxic cultures, but the nature of the toxin could not be determined by a toxin-antitoxin test.

In November, 1921, Dr. J. C. Geiger collected 20 samples of virgin (?) soil from the Ozark Mountains, Government Reservation, Hot Springs, Ark. Two cultures were weakly toxic, 1 contained *B. tetani* and 4 produced "malignant edema."

Summary: 8 cultivated soils or animal corrals, 1 weak toxin, 20 virgin soil, 2 weak toxin, 13 feeds, vegetables, 1 type B; 2 weak toxin. Total: 41 with 1 (6), or 2.4 (14.8) % positive cultures.

Colorado.—Three human outbreaks (2 caused by commercially canned string beans or spinach grown and packed in the state of Kansas; 1 home canned beets grown and packed near Pueblo); 3 chicken outbreaks due to home canned vegetables (2 corn, Fort Collins, and 1 string beans, Lyons).

Mr. R. S. Hiltner, Director, Colorado Inspection Service, of the National Cannery Association, Denver, procured, in December, 1920, from Brighton (4,970 ft.) and Longmont (4,935 ft.), Colorado, a total of 27 samples consisting of soils (12), tomato plants and roots (4), bean plants, vines and roots (8), pumpkin and cucumber vines and roots (3) were cultures; one culture, prepared from a wax bean plant (East of Longmont), was toxic, but the nature of the toxin could not be determined by a toxin-antitoxin test.

In December, 1921, Inspector M. M. Allison collected 3 sets of soil and plant remnants from farms near Fowler, Colo., in the Arkansas Valley, about 40 miles east of Pueblo. The farms were irrigated by ditches from the Arkansas River. Nine cultures, prepared from soils (3), tomato stems, leaves and roots (2), bean stalks and roots (2) and cucumber vines and roots (2), gave the following results:

Soil, tomato field	} B. botulinus, type A.
Soil, bean field	
Bean stalks	
Bean roots	

Tomato roots and vines from cucumber fields produced weakly toxic cultures.

Dr. I. E. Newsom, Department of Pathology, Colorado Agricultural College and Experiment Station, Fort Collins, submitted for examination a specimen of dried crop content of a chicken (Lyons) and 5 samples of moldy alfalfa and dry beet pulp (feed lot, Ault sugar farm near Greeley). The crop specimen contained *B. botulinus*, type A (for details see Chicken Report 41). In April, 1922, a specimen of home canned corn and the crop content of a chicken, which contained *B. botulinus*, type A, was received from the same laboratory.*

In Sept., 1921, Drs. Oscar I. Kron and F. M. Hayes collected 14 virgin soil samples from Pike's Peak (at an elevation of 11,000 ft.), 5 samples from Cheyenne Canyon, 4 from Mushroom Park, Garden of the Gods, and 4 from the Cave of the Winds. The cultural findings were:

Pike's Peak, 4—*B. botulinus*, type A; 2 weak toxins.

Cheyenne Canyon, 1—*B. botulinus*, type A.

Mushroom Park, 1—*B. botulinus*, type A.

Cave of the Winds, 1—*B. botulinus*, type A.

Summary: 32 cultivated soils and its products; 1 weak toxin; 9 irrigated soils and its products; 4 type A; 2 weak toxins; 27 virgin mountain soil; 7 type A; 2 weak toxins.

Total: 68 samples with 11 (16), or 16.1 (25.0) % positive cultures.

One crop specimen (home canned string beans) type A; 1 home canned corn and 1 crop specimen, type A.

Connecticut.—In Sept., 1921, Mr. C. J. Mason, Instructor in Bacteriology, Connecticut Agricultural College, Storrs, collected and submitted for examination, 9 samples of soil (poultry yard, pig pasture [2], grass field [2], clover field, cattle corral, pig pen, corn field) and 15 vegetable specimens (beets, potatoes, carrots [2], peas, musty straw, beet greens, corn stalks, turnip tops, decayed tomatoes, cucumbers, bean stalks, bean pods, musty hay, mangel tops). The beets furnished a type A, *B. botulinus*, while the potatoes, musty straw, corn stalks, decayed bean stalks and tomatoes gave weakly toxic cultures.

Summary: 9 cultivated or manured soils, 15 vegetables, feed, etc.—1 type A, 5 weak toxins. Total: 24 samples with 4.1 (25) % positive cultures.

Delaware.—Mr. F. C. Blanck, Director of Inspection, National Cannery Association, Easton, Md., collected and forwarded, in December, 1920, from different points in Delaware, 6 samples of soil (lima bean, tomato, red pepper,

string bean, bush bean and sweet potato fields) and 12 specimens of leaves and roots from the fields mentioned. The following cultures were toxic:

Soil, husks and leaves from string bean field, 2 *B. botulinus*, type B.

Soil, tops and leaves from tomato field; 2 *B. botulinus*, type B.

Lima beans and bush bean husks, weak toxins.

Summary: 6 cultivated soil, 2 type B. 12 plant products, 2 type B and 2 weak toxins.

Total: 18 samples with 4 (16), or 22.2 (33.3) % positive cultures.

District of Columbia.—One chicken outbreak, due to corn, no earth samples.

Florida.—One human outbreak at Tampa due to home preserved ham.

In November, 1921, Dr. J. C. Geiger collected, near Miami, 20 samples of unfertilized soil. One culture contained a weak toxin. At the same time, Dr. W. G. W. Ells, Assistant Plant Pathologist, Agricultural Experiment Station, University of Florida, Gainesville, collected and forwarded 25 duplicate samples of soils and decayed plants from the vicinity of the experiment station. Cultures were made from soil from cattle corral, horse corral, cane field, citrus orchard, garden, corn field, hog lot, chicken run, muck soil, peat, clay, barren field, dirt road, edge of pond, from under yellow pine tree, shrub land, potato land, Bermuda sod, meadow (Natal grass), cherry laurel hedge, virgin soil from hammock, oak and hickory, yellow pine land and large oak land and decomposed roots and leaves. Eight cultures were positive, namely:

Decomposed roots and leaves, 2—*B. botulinus*, type B.

Soil from citrus orchard, 1—*B. botulinus*, type B.

Soil from meadow, Natal grass, 1—*B. botulinus*, type B.

Muck soil, 1—*B. botulinus*, type B.

Soil from edge of a pond, 1—*B. botulinus*, type B.

Soil from ordinary, poor blackjack shrub land, 1—*B. botulinus*, type B.

Soil from cane field, 1—*B. botulinus*, type A.

Summary: 43 soils from diverse sources, 5 type B, *B. botulinus*, 1 type A, 1 weak toxin; 2 decomposed plants, 2 type B.; 45 with 8, or 17.7% positive cultures.

Georgia.—In May, 1921, Dr. B. B. Higgins, Botanist, Georgia Experiment Station, Experiment, Ga., collected and submitted for examination 20 samples of soils and its products: soil from pasture, cow lot, corn stubble, clover land, chicken yard, hog lot and virgin soil, moldy hay (2), bean pods and stalks, beans and vine, cabbage (2), peas, cornstalk, onions, daffodil bulbs, turnips, English garden peas, beet root. Five cultures gave relatively strong toxins:

Moldy hay, 1—*B. botulinus*, type B.

Bean pods and stalks, 1—*B. botulinus*, type B.

Beans and vines, 1—*B. botulinus*, type B.

Decayed cabbage, 1—*B. botulinus*, type B.

Turnips, 1—*B. botulinus*, type B.

Summary: 7 soil samples, all negative; 13 vegetables and feeds, 5 type B.

Total: 20 samples with 5, or 25%, positive cultures.

Idaho.—Three human outbreaks (1 locally grown and home canned asparagus, Boise, 1 locally grown and home canned greens and turnip tops, Cambridge, and 1 locally grown and home canned corn, Weiser).

In November, 1921, Professor William M. Gibbs, head of the Department of Bacteriology, College of Agriculture and Agricultural Experiment Station, University of Idaho, Moscow, collected and forwarded for examination 32 samples of soils and vegetables and feeds: soil from turnip field, chicken yard, cattle pasture, manure, pea field, corn field, sheep corral, cattle corral (2), pig

pen, orchard, grain lot, subsoil excavation, virgin soil (3), turnip tops (2), grass from pasture, corn stalk, peas and roots, corn, decomposed silage, larch leaves (Arboretum), cabbage, apples, pears and cucumbers rotting on ground, beet tops, beets dug from ground, chard and parsnips dug from ground.

Three cultures were highly toxic, while 3 produced symptoms, but could not be identified. The following samples furnished these cultures:

Virgin soil under larch growth, *B. botulinus*, type A.

Cucumber rotting on ground, *B. botulinus*, type A (isolated).

Beet tops, beets still growing, *B. botulinus*, type B.

Soil from turnip field, cornstalk and beets, dug from ground gave weak toxins.

In February, 1922, Dr. W. R. Hamilton, Weiser, sent a jar of locally grown and packed home canned corn belonging to the same lot, which caused the death of one child at Weiser, Report 99. The corn was toxic and *B. botulinus*, type A, was isolated.

Summary: 15 soil samples, 1 virgin soil, type A; 18 vegetables and feeds and home canned plant products, 2 type A and 1 type B, 3 weak toxins.

Total: 33 samples with 4 (7), or 12.1 (21.2)%, positive cultures.

Illinois.—One incompletely proved human outbreak at Chicago, one chicken outbreak, due to garbage, one outbreak among horses caused by ensilage.

The Hoopston Canning Company submitted, in December, 1920, 2 samples of soil collected from heavily manured corn fields and 2 specimens of sweet corn roots; 8 cultures were prepared and one of each soil specimen contained a weak toxin, producing symptoms and death in 10 days.

The Gibson Canning Company, Gibson City, furnished, in December, 1920, one soil sample and 5 specimens of sweet corn stalks, husks, leaves, etc. One culture prepared with the corn stalk contained a toxin which was fatal to a guinea-pig on the twenty-sixth day.

Mr. David M. Morgan of Mound City forwarded 5 samples of soil (sweet corn, tomato, sweet potato, hominy, corn and pumpkin fields) and 10 specimens of roots, leaves, vines, etc., collected from the same areas in December, 1920. Ten specimens of the cultures prepared with the sweet corn and sweet potato leaves contained *B. botulinus*, type B.

The Interrieden Canning Company, Grayslake, forwarded 5 soil specimens and 7 samples of corn leaves, husks, pea vines, roots and leaves collected on their farms, in December, 1920. The cultures prepared with these specimens were all nontoxic.

The George E. Stocking Canning Factory, Rochelle, supplied 2 specimens of soil derived from a pea field together with some leaves of a well cured stack. The samples furnished nontoxic cultures.

Summary: 14 soils from pea, corn, tomato, etc., fields, 2 weak toxins; 25 leaves, husks, vines, etc., 2 type B, *B. botulinus*, and 1 weak toxin.

Total: 39 samples with 2 (5), or 5.1 (12.8) %, positive cultures.

Indiana.—Two human outbreaks (one due to locally grown and home canned string beans and one due to commercially packed spinach from California, at Kendallville).

Mr. John P. Street, Director of Indiana Inspection Service, National Canners Association, Indianapolis, procured 1 sample of soil and 2 specimens of roots and stems from a tomato field at Paoli, and 3 similar specimens from Red Key. These samples furnished nontoxic cultures.

Dr. L. P. Doyle, Purdue University, Agricultural Experiment Station, Lafayette, collected and submitted for examination 7 samples of soil from cattle pen, pig pen, poultry yard, corn field, wheat field, clover field and

pasture, and 15 specimens of vegetables and plants: corn stalk, decaying vegetables (2), straw, moldy straw, beanstalks, bean pods (2), pea vine, swiss chard, beet top, turnips, beets, carrots and radishes. The following cultures were toxic:

Soil corn field, 1—*B. botulinus*, type B.

Soil wheat field, 1—*B. botulinus*, type B.

Soil clover field, 1—*B. botulinus*, type B.

Soil pasture, 1—weak toxin and *B. tetani*.

Bean stalks and pods, 2—*B. botulinus*, type B, 1 weak toxin.

Radishes, 1—weak toxin.

Swiss chard, *B. tetani*.

Summary: 9 soils from cultivated fields and pasture, 3 type B, 1 weak toxin and *B. tetani*; 19 vegetables and feeds, 2 type B, 2 weak toxin and *B. tetani*.

Total: 28 samples with 5 (8), or 17.8 (28.4) %, positive cultures.

Iowa.—One unproved human outbreak, due to home prepared ham and salted pork, at Sioux Rapids.

Mr. William H. Harrison, Director of the Iowa Inspection Service, National Cannery Association, collected and forwarded, in December, 1920, one sample of soil and one specimen each of corn leaves, husks and roots on a farm near Altoona. Similar sets of specimens were taken at Grimes (about 14 miles northwest of Des Moines) and at Vinton.

Dr. Charles Murray, Division of Veterinary Medicine, Iowa State College, Ames, collected, in September, 1921, and forwarded for study 8 samples of soil (hog yard, horse corral, cattle corral, poultry yard, corn field, clover field, oat field, pasture) and 14 specimens of vegetables and feeds: oats (2), beets, carrots, turnips, beans and pods, decayed straw, cabbage, radish roots and tops, moldy hay, corn stalks, decayed vegetables (2).

One sample of oats gave a culture containing *B. botulinus* type A, while those prepared from the radish roots and carrots were weakly toxic.

Summary: 11 cultivated soils, all negative; 23 vegetables and feeds, 1 type A, 2 weak toxins.

Total: 34 samples with 1 (3), or 2.9 (5.8) %, positive.

Kansas.—Mr. H. R. Baker, Department of Bacteriology, Kansas State Agricultural College, Manhattan, collected and forwarded, in May, 1921, 10 samples of soil (horse corral [2], cow pasture, cow corral, pig pen, sheep corral, poultry yard, grain field, clover field and corn field fertilized with sheep manure) and 20 specimens of vegetables and feeds: bottom of a Sudan grass straw stack, decayed straw (2), corn stalks, rotted, frost bitten alfalfa, young green mangels, young turnips, decayed leaves in barnyard, spinach, straw, beets, string beans, carrots, radishes, beet tops, pea stalks, decayed vegetation, and bean stalks and lettuce (2).

Beets bought on the market, *B. botulinus*, type A.

String beans bought on the market, *B. botulinus*, type A.

Summary: 10 cultivated or manured soils, all negative; 20 vegetables and feeds, 2 type A.

Total: 30 samples with 2, or 6.6%, positive cultures.

Kentucky.—One outbreak among mules, due to ensilage prepared in Carroll County.

Dr. A. J. Steiner, Department of Veterinary Science, Agricultural Experiment Station of the College of Agriculture, University of Kentucky, Lexington, collected and submitted, in June, 1921, 7 samples of soil (hog pen, dairy pasture, manure and soil from cow run, chicken run, corn field [2], rye field)

and ten specimens of vegetables and feeds: asparagus roots, potato, wheat heads, lettuce, spinach, onions, beets, corn stalks, soja beans and radishes. The following specimens furnished highly toxic cultures:

Soil from hog pen on college farm (has been used as such for many years, *B. botulinus*, type B.

Soil from dairy pasture, *B. botulinus*, type B.

Soil from field that contained clover last year, corn this year, *B. botulinus*, type B.

Soil from plot which has been planted in corn for the last seven years, *B. botulinus*, type B.

Radishes, onions, beets and lettuce from Dr. D.'s garden, 4 *B. botulinus*, type B.

Potato from College farm garden, *B. botulinus*, type B.

Wheat heads from College farm, *B. botulinus*, type B.

Corn stalks from Experiment Station Farm, *B. botulinus*, type A.

Soja beans from Experiment Station Farm, *B. botulinus*, type B.

Summary: 7 cultivated or manured soils, 4 type B; 10 vegetables and feeds, 7 type B, 1 type A.

Total: 17 samples with 12, or 70.5%, positive cultures.

Louisiana.—In March, 1921, Dr. Harry Morris, Bacteriologist, College of Agriculture and Agricultural Experiment Station, Louisiana State University, Baton Rouge, collected around the University Campus and Experiment Station 8 samples of soil (cattle lot, hog lot, chicken yard, oat field, sugar cane field, pasture, corn field, alfalfa field) and 20 specimens of plant products: mustard, beets, decaying rice straw (2), moldy soja bean hay, lettuce, pea pods and stalks, soja beans, decaying onions, beet tops, green beans, decaying sweet potatoes, decaying leaves, carrot tops, carrots, decaying cabbage leaves, corn stalks (2), husks and corn. The following cultures were toxic:

Corn stalks, *B. botulinus*, type A.

Moldy soja bean hay, *B. botulinus*, type A.

Lettuce, beet tops, green beans and corn furnished weakly toxic cultures.

In November, 1921, Dr. J. C. Geiger collected in the foothills 5 miles north of Alexandria, 20 samples of soil from cut over timberland used for grazing purposes. The cultures were all nontoxic.

Summary: 8 cultivated soils, all negative; 20 timberland, used for grazing, all negative; 20 vegetables and feeds, 2 type A, 4 weak toxins.

Total: 48 samples with 2 (6), or 4.1 (12.5) %, positive cultures.

Maine.—One human outbreak due to commercially prepared ham at a summer resort.

Miss F. L. Chandler, Department of Bacteriology and Veterinary Science, University of Maine, Orono, collected and forwarded, in September, 1921, 7 samples of soil (corn field, clover field, hen yard, cow yard, pig pen, pasture, grain field) and 14 specimens of vegetables and plants (cucumbers, lettuce, decaying vegetables, tomatoes, cabbage, beets and tops, beans, carrots, rape, corn stalks, moldy hay, turnips, spoiled silage, pea pods and stalks). The following cultures were highly toxic:

Lettuce, cabbage, moldy hay, pea pods and stalks, 4 *B. botulinus*, type A.

Beans and turnips, 2 *B. botulinus*, type B.

In October, 1921, Dr. J. C. Geiger collected from the foothills (uncultivated section) near Bath, Lewiston, Augusta and Poland Springs 20 samples of virgin forest soil. Four cultures made from red soil were highly toxic and contained *B. botulinus*, type A.

Summary: 7 cultivated soil, all negative; 20 virgin forest soil, 4 type A; 14 vegetables and feeds, 4 type A, 2 type B.

Total: 41 samples with 10, or 24.3%, positive cultures.

Maryland.—Mr. F. C. Blanck, Director of Inspection, National Canners Association, Easton, collected and forwarded, in December, 1920, 2 samples of soil (tomato and corn fields) and 4 specimens of stems and roots procured from the same fields. The cultures prepared from the soil of the corn field contained *B. botulinus*, type A, while those made with soil of the tomato field and tomato roots were not sufficiently toxic to permit identification.

In February, 1921, cultures were made of 4 samples of hog manure obtained at Perryville, with negative results.

Dr. J. C. Geiger collected 20 samples of virgin soil about 40 to 50 miles from Washington, D. C., in the foothill region of the Blue Ridge Mountains. Eleven cultures contained *B. botulinus*, type B, 2 type A and B, and 1 *B. botulinus*, type B, and *B. tetani*.

Summary: 2 cultivated soil, 1 type A, 1 weak toxin; 20 virgin soil, 11 type B, 2 type A and B, 1 *B. tetani* and *B. botulinus*; 4 vegetables, 1 weak toxin; 4 hog manure, all negative.

Total: 30 samples with 15 (17), or 50 (56.6) %, positive cultures.

Massachusetts.—Three human outbreaks (one due to minced chicken, one due to locally prepared blood sausage, at Lowell, one cause unknown).

Mr. J. Raymond Sanborn, Department of Microbiology, Massachusetts Agricultural College, Amherst, collected and forwarded, in April, 1921, 5 samples of soil (clover field, poultry yard, cow corral, pasture land and corn field) and 12 specimens of vegetables and feeds: parsnips, cabbage stubs, moldy hay (2), corn stalks (2), carrots, dandelion, lettuce, beans, soja beans and decaying corn stalks. The soil from the pasture land contained *B. botulinus*, type B, while the parsnips and beans produced weak toxins.

Summary: 5 cultivated and manured soils, 1 type B; 12 vegetables and feeds, 2 weak toxins.

Total: 17 samples with 1 (3), or 5.8 (17.0) %, positive cultures.

Michigan.—Three human outbreaks due to olives and spinach commercially packed in California.

Mr. Frank Gerber, of the Fremont Canning Company, collected, in November, 1920, and forwarded through Mr. A. B. Todd, Director of Inspection for Michigan, National Canners Association, 24 samples of soils (6 specimens each from pea fields, corn fields, Michigan bean fields, and cabbage fields) and 24 specimens of pea vines, corn husks and white bean vines and roots.

One sample each of the corn husks, white bean vines, pea vines and soil of corn garden produced weak toxins.

In July, 1921, Mrs. Zae Northrup Wyant, Research Associate in Bacteriology, Michigan Agricultural College, East Lansing, collected and submitted for examination 28 samples of soil: newly cultivated bean field, bean field, oat field, corn garden, pea garden, potato garden, clay soil from garden, soil from garden (2), soil from newly plowed field, soil from under mulch on strawberries, soil from barnyard, soil from vineyard, soil from 7 pens poultry house, pig pen, yard, alfalfa field, pea patch, cucumber patch, barnyard, timothy field, corn field, bean field, potato field, wheat field, garden soil, pole bean garden, poultry ranch, 5 specimens of silage (from bottom of Bacteriology barn silo); 5 samples of feeds (lettuce, new timothy and clover hay, dried beet pulp, ear corn, corn flakes) and 14 samples of manure and compost. The following cultures were positive:

Silage northwest silo, Dairy barn, 2 feet deep only; *B. botulinus*, type B (48 hour toxin).

Soil from underneath mulch on strawberries, College Farm, *B. botulinus*, type B.

Soil and bedding from pig pen and yard, west end of barn, weak toxin.

Chicken manure (Wyant); *B. botulinus*, type A.

In September, 1921, Dr. J. C. Geiger obtained 10 samples of virgin soil from wooded areas (20 to 30 miles from Detroit) and 10 samples of garden soil from the Tuberculosis Farm near Detroit. Two cultures prepared with garden soil contained *B. botulinus*, type B, and 2 other cultures *B. tetani*.

Summary: 82 fertilized or manured soils, 3 type B, 2 weak toxins, 2 *B. tetani*; 10 virgin soil, all negative; 5 silage, 1 type B; 29 vegetables and feeds, etc., 3 weak toxins; 14 manure and compost, 1 type A.

Total: 120 samples with 5 (10), or 4.1 (8.3) %, positive cultures.

Minnesota.—The Minnesota Valley Canning Company, Le Sueur, submitted in December, 1920, 4 samples of soil (corn ground [2] and pea fields [2]) and 4 specimens of corn husks, pea plants and roots. The cultures prepared with these samples were all negative. Additional samples of soil (4) and plant products (4) were sent from other farms of the same company. The cultures were nontoxic.

Mr. C. D. Geidel, Director of the Minnesota Inspection Service, National Canners Association, St. Paul, forwarded in January, 1921, 6 samples of soil (3 different farms) and 4 specimens of corn roots and corn leaves collected by the Barr Pickling and Preserving Company of St. Cloud. One culture of the upland soil, Barr Farm, contained *B. botulinus*, type A, while 2 other soil specimens produced weak toxins.

In October, 1921, Dr. J. C. Geiger collected 10 samples of virgin soil from Minnehaha Creek in the vicinity of Minneapolis and 10 samples of soils from vegetable gardens. One culture of garden soil contained *B. botulinus*, type A and one specimen of virgin soil produced a weak toxin. Two garden soils contained *B. tetani*.

Summary: 24 cultivated or manured soils; 2 type A and 2 weak toxins; 10 virgin soil, 1 weak toxin; 12 roots, leaves, vegetables, etc., all negative.

Total: 46 samples with 2 (5), or 4.3 (10.8) %, positive cultures.

Mississippi.—Dr. Charles F. Briscoe, Bacteriologist, Mississippi Agricultural and Mechanical College, Agricultural College, collected and forwarded, in May, 1921, 6 samples of soil (cow stable, pig pen, poultry pen, clover field, unknown [2]) and 13 specimens of vegetables, feeds, roots, etc.: decaying vegetation (2), bean pods and stalks, moldy hay (2), pea pods and stalks, corn stalks, turnip roots, radish tops, beet roots, carrot roots, fresh lettuce and beet tops. The following cultures were toxic:

Decaying vegetation, *B. botulinus*, type B.

Corn stalks, *B. botulinus*, type B.

Turnip roots, *B. botulinus*, type B.

One sample each of soil, moldy hay and carrot roots gave weakly toxic cultures.

Summary: 6 cultivated or manured soils, 1 weak toxin; 13 vegetables, feeds, etc., 3 *B. botulinus*, type B, 2 weak toxins.

Total: 19 samples with 3 (6), or 15.7 (31.5) %, positive cultures.

Missouri.—Prof. William A. Albrecht, Department of Soils, College of Agriculture, University of Missouri, Columbia, collected and forwarded, in March, 1921, 7 samples of continuously cropped and manured soil (blue grass

pasture, hog lot, rye field, cattle lot, chicken yard, corn lot, clover field) and 7 specimens of vegetables, compost, etc. (ensilage [2], corn stalks, decaying blue grass hay, leaf compost, blue grass manure, fresh lettuce) and jars of spoiled home canned beans. The following cultures were toxic:

Soils from blue grass pasture, hog lot and corn field, 3 *B. botulinus*, type B.

Ensilage fresh collection, 1 *B. botulinus*, type B.

Decaying blue grass hay, 1 *B. botulinus*, type B.

Manure blue grass, 1 *B. botulinus*, type B.

Home canned beans (nontoxic brine), 1 *B. botulinus*, type A.

One of us (B. J. D.) collected, in July, 1921, near the Campus of the University of Missouri, 10 samples of soil (street, driveway, clay soil, top soil, turned up soil near pond, walk near dairy), wheat (2), and manure (2), and 7 specimens of vegetables, feeds and manure (decomposed hay [2], wheat [3], manure [2]). The following cultures were toxic:

Soils, near pond, near dairy, wheat field (2), 4 *B. botulinus*, type B.

Two soil samples and one specimen of wheat gave weak toxin.

In June, 1921, the laboratory received from Peruque, two small wild ducks. The cadavers were badly decomposed, but coccidia were found in the liver and intestines. Cultures were taken from a composite sample of the intestinal tract and *B. botulinus*, type B was demonstrated in these specimens.

Summary: 17 soils continuously cropped and manured, etc., 7 type B and 2 weak toxins; 14 vegetables, ensilage, manure, 3 type B and 1 weak toxin; 1 intestines of wild ducks, 1 type B; 2 home canned string beans, 1 type A.

Total: 34 samples with 12 (15), or 35.2 (44.1) %, positive cultures.

Montana.—One human outbreak at Java, due to stuffed ripe olives grown in California.

Dr. D. B. Swingle, Department of Botany and Bacteriology, Agricultural Experiment Station, University of Montana, Bozeman, collected and forwarded, in September, 1921, 9 samples of soil and dirt (rabbit yard, clover field, corn field, chicken pen, pig corral, sheep pen, horse pen, grain field, duck pen) and 16 specimens of vegetables, manure, silage, etc. (carrots, moldy alfalfa, beans, decayed beets, rutabaga, peas, summer squash, lettuce, corn silage, tomatoes, crab apple, cabbage leaves, parsley, spinach and cucumbers, cow manure). The following cultures were toxic:

Carrots, lettuce, corn silage, 3 type A.

Moldy alfalfa, 1 type A and B.

Dirt, horse pen, 1 type B.

Parsley, green tomatoes, rutabaga and summer squash, 4 weak toxins.

Dr. J. C. Geiger collected, in September, 1921, 41 samples of soil from 3 different areas in Glacier National Park. The cultures gave the following results:

Area 1: West side of Continental Divide along Lake Ellen Wilson and Gunsight Pass—12 samples, 5 type A.

Area 2: East side of Continental Divide, foot of Swift Current Pass and Mt. Wilbur—16 samples, 2 type A and 2 type B, 2 weak toxins.

Area 3: Foothills of eastern side of Continental Divide (Midvale Creek and Squaw Mountain)—13 samples, 2 type A and 5 weak toxins.

Summary: 9 soils or dirt from manured places, 1 type B; 41 virgin mountain soils, 9 type A, 2 type B; 7 weak toxins; 16 vegetables, feeds, etc., 3 type A, 1 type A and B, 4 weak toxins.

Total: 66 samples with 16 (27), or 24.2 (40.9) %, positive cultures.

Nebraska.—Dr. L. Van Es, Department of Animal Pathology and Hygiene, University of Nebraska, Lincoln, forwarded, in May, 1921, 12 samples of soil (poultry yard, cow yard, sheep yard, cattle yard, hog yard, wheat field, corn field, field No. 8, small bull pasture, hog yard (serum plant), horse yard, alfalfa field) and 11 specimens of vegetables, feeds, etc. (bottom of old hay stacks [2], corn stalks, corn silage, lettuce, alfalfa, decayed cabbage stalks, old manure pile [2], dandelions, old beets from root cellar). The following cultures were toxic:

Old manure pile (Havelock), 1 *B. botulinus*, type A.

Dandelions, 1 *B. botulinus*, type A.

Soil, poultry yard, hog yard, wheat field, alfalfa field, gave weak toxins.

Summary: 12 cultivated or manured soils, 4 weak toxins; 11 vegetables and manure pile, 2 type A.

Total: 23 samples with 2 (6), or 8.6 (26.0) % positive cultures.

Nevada.—Five chicken outbreaks, due to home canned string beans (1), moldy potatoes (2), spoiled sour milk (1) and spoiled canned goods (1).

Dr. J. C. Geiger brought, in December, 1920, 3 samples of field soil and of garden soil from the vicinity of Reno and 2 samples of garden and 2 of desert soil from Hastings. One sample of garden and one of desert soil produced weakly toxic cultures. The remainder was nontoxic.

Mr. G. D. Delprat, Jr., collected in Sage County near the California border 3 samples of virgin soil. Two contained *B. botulinus*, type A.

Dr. L. R. Vawter, University of Nevada, forwarded, in December, 1921, 11 samples of soil. The cultures gave the following results:

Virgin soil along road through secret pass in the Ruby Mountains (7,000 feet)—1 type A (south of Elko).

Sage brush bench, north of Elko, used as a range for cattle and sheep—1 type A.

Creek bed soil, Gardnerville—1 type A.

Soil alfalfa field, Truckee Valley, irrigated 8 miles south of Reno—1 type A.

Virgin sage brush soil at foot of Mt. Rose (20 miles southwest of Reno)—1 type A.

Lake bed soil, bank of Little Washoe Lake, in Washoe Valley—1 type A.

Garden soil in Reno (avian botulism), hayfield, and river bank adjoining Humboldt River, Elko, garden soil in Gardnerville and Steamboat Springs, Geyser Basin—all negative.

Summary: 5 field soil, 1 *B. botulinus*, type A; 8 garden soil, 1 *B. botulinus*, type A, 1 weak toxin; 4 desert and sage brush, 2 *B. botulinus*, type A, 1 weak toxin; 8 virgin soil, 5 *B. botulinus*, type A.

Total: 25 samples with 9 (11), or 36.0 (44.0) %, positive cultures.

Several isolations of *B. botulinus*, type A and B from the livers of cattle by Dr. L. R. Vawter should be added.

New Hampshire.—Prof. Mabel Brown, New Hampshire Agricultural Experiment Station, Durham, collected and forwarded 6 samples of soil (cattle corral, poultry yard, oat field, corn field, pig pen, clover field) and 8 specimens of vegetables and feeds (radishes, bean stalk, pea stalk, beets, decaying leaves, lettuce, hay and pea pods).

The sample of hay contained *B. botulinus*, type B; the pea stalk gave a weak toxin.

Summary: 6 cultivated or manured soils, all negative; 8 vegetables and feeds, 1 *B. botulinus*, type B, 1 weak toxin.

Total: 14 samples with 1 (2), or 7.1 (14.2) %, positive cultures.

New Jersey.—One human outbreak, due to home canned string beans, at Newark.

Dr. J. H. McNeil, Chief, Bureau of Animal Industry, Department of Agriculture, Trenton, forwarded, in February, 1921, 5 samples of soil collected from salt meadows near Toms River and Osbornville, eastern section of the state. One sample from J. Farm, one from Westervelt and one from Johns Farm contained *B. botulinus*, type B.

Dr. Fred Boerner, Jr., Bureau of Animal Industry, Pennsylvania Department of Agriculture, Philadelphia, collected and forwarded, in February, 1921, 20 samples of soil and feeds from 8 different localities. The following results were obtained with the cultures:

Soil and sod from salt meadow, Cape May, 2 *B. botulinus*, type B.

Soil, mixture of hay (1), hay, Swainton, 1 weak toxin.

Soil and corn, South Dennis, all negative.

Soil (2), forage alfalfa, corn, woodbine, all negative (4).

Soil, corn, salt hay, corn stover, near Goshen, all negative.

Soil, Dias Creek, negative (1).

Soil, feed mixture, corn stover, salt hay, all negative (4).

Soil, Rio Grande, 1 weak toxin.

Two samples of soil collected from tomato field near Camden were cultivated, in February, 1921, and found to be free from *B. botulinus* spores.

Dr. F. G. Steinbach of Wildwood submitted, in October, 1921, 3 samples of soil (H. and F. Farms) one of manure (1), and 4 of feeds (alfalfa, moldy bread, corn stubble and stover). The cultures gave the following results:

Soil H. farm, 1 *B. botulinus*, type A.

Soil F. farm, 1 *B. botulinus*, type B.

Summary: 20 meadow soils and sod from salt farms, 6 type B, 1 type A, 2 weak toxins; 1 manure, negative; 15 feeds, hay corn, etc., all negative.

Total: 36 samples with 7 (9), or 19.4 (25) %, positive cultures.

New Mexico.—Mr. Harold Gray, State Engineer, Santa Fe, forwarded, in December, 1920, 8 samples of soil supposedly collected in various sections of the state. The cultures prepared from these samples were all nontoxic.

New York.—Five human outbreaks, one in New York City, due to ripe olives grown and packed in California, one to home prepared cottage cheese in the western part of the state, one to home canned corn, one to commercially packed spinach and one to unknown food products, the last two in New York City.

S. K. Farrar, Assistant Director of Inspection, National Canners Association, Rochester, forwarded, in January, 1921, 3 samples of soil (Red Creek and Oswego) and 4 specimens of cabbage leaves, corn husks and pea vines. The cultures gave the following results:

Soil, Oswego, *B. botulinus*, type A.

Cabbage from field near Red Creek, *B. botulinus*, type A.

Peavine, weak toxin.

Dr. George A. Stock, Medical Examiner, U. S. Veterans Bureau, forwarded, in October, 1921, 24 samples of virgin soil collected from Mount Baker, which is in the vicinity of Saranac Lake. Four samples contained *B. botulinus*, type A. 1 gave a weak toxin and 3 contained *B. tetani*.

Summary: 3 cultivated and manured fields, 1 type A; 24 virgin soils, 4 type A, 1 weak toxin; 4 vegetables, etc., 1 type A, 1 weak toxin.

Total: 31 samples with 6 (8), or 19.3 (25.8) %, positive cultures.

North Carolina.—Dr. F. A. Wolf, Department of Botany, North Carolina Agricultural Experiment Station, West Raleigh, collected and forwarded, in

April, 1921, 12 samples of soil (cow pen, vineyard, garden, cattle and sheep pasture, oat field, pig pen, apple orchard and chicken pen, clover field and corn field, sheep pen and horse pen) and 8 specimens of vegetables and feeds (cabbages, peas, lettuce, chard, onions, corn stalks, beans and radishes). The following cultures contained weak toxins, which could not be identified by toxin-antitoxin tests: soil from vineyard, garden, cattle pasture, radishes, chard and onions.

Summary: 12 cultivated soils, 3 weak toxins; 8 vegetables, 3 weak toxins. Total: 20 samples with 6, or 30%, toxic cultures.

North Dakota.—Dr. A. F. Schalk, North Dakota Agricultural College, collected and forwarded, in May, 1921, 7 samples of soil (cattle corral, poultry yard, corn field, clover field, pig pen, pasture, grain field) and 7 specimens of vegetables and feeds (radishes, moldy straw, forage, corn stalks, decaying roots [2] and lettuce).

The culture prepared with the forage contained *B. botulinus*, type A, while those made from the moldy straw and corn stalks were weakly toxic.

Summary: 7 cultivated soils, all negative; 7 vegetables and feeds, 1 type A, and 2 weak toxins.

Total: 14 samples with 1 (3), or 7.1 (21.4) %, positive cultures.

Ohio.—Two human outbreaks, at Canton, one due to ripe olives packed in California and one at Canaan, due to spinach.

The Sears Nichols Canning Company at Chillicothe submitted, in June, 1920, 2 samples of beets and soil. Six cultures were prepared and found to be nontoxic. In October, 1920, the same company forwarded 5 samples of soil (beet field [2], lima bean field, sweet corn field and spinach field) and 7 specimens of beets, lima beans, green corn and raw spinach. A sample of soil from the sweet corn field contained *B. botulinus*, type B.

Mr. A. M. Wadsworth, Director of Inspection, National Canners Association, Columbus, forwarded, in December, 1920, from Canal Winchester, 6 samples of soil and 8 specimens of vegetables and plant remnants from pea, lima bean and corn fields.

One sample of lima beans from G. B. farm contained *B. botulinus*, type B. One sample of corn husks from C. A. farm produced a weak toxin.

M. A. Bates, Assistant Director of Inspection, National Canners Association, Columbus, forwarded, in December, 1920, 5 samples of soil and 8 specimens of vegetables and plant remnants from a cabbage field near Fremont and Bellevue, corn and strawberry fields near Elyria, tomato fields near Madison and corn fields near DeGraff. A culture prepared with cabbage leaves from Bellevue contained *B. botulinus*, type B and another from Fremont produced a weak toxin.

Summary: 18 cultivated soils, 1 type B; 23 vegetables or plant remnants, 2 type B and 2 weak toxins.

Total: 41 samples with 3 (5), or 7.3 (12.1) %, positive cultures.

Oklahoma.—Dr. L. L. Lewis, Oklahoma Agricultural and Mechanical College, Stillwater, forwarded, in April, 1921, 10 samples of soil (one-half to 5 inches depth, barnyard, poultry, sheep, cattle yard, hog lot) and 14 specimens of vegetables and plant remnants (decaying canna tops, decaying cauliflower, cinari, spinach tops, lettuce, tomatoes, sweet potatoes, radish roots, horse radish, onion roots, pea tops, decayed wheat straw, salsify roots and decayed cane hay). One culture prepared from decaying canna tops contained *B. botulinus*, type A. Horse radish, decayed cane straw and pea tops gave weak toxins.

Summary: 10 cultivated or manured soil, negative; 14 vegetables and plant remnants, 1 type B, 3 weak toxins.

Total: 24 samples with 1 (3), or 4.1 (12.5) %, positive cultures.

Oregon.—Four human and 2 fowl outbreaks; one at Hillsboro, due to home canned corn, one at Ontario, one at Klamath Falls and one at Grants Pass, all due to home canned string beans; 2 fowl outbreaks were caused by home canned string beans at Klamath Falls.

In August, 1920, E. Wagner collected and brought to the laboratory from Ashland, 3 samples of soil (barnyard, strawberry bed, pea garden); 2 specimens of fruits from the trees (cherries, bird pecked and sound) and 7 specimens of vegetables (beets, peas, and sugar peas, asparagus, bean vines, moldy vines, leaf mold, strawberries). The following results were obtained:

Sugar peas, leaf mold and asparagus tips contained *B. botulinus*, type A.

Soil from pea garden, barnyard and asparagus, beets, pea vines, moldy leaves (2), bean vine and cherries gave 9 weakly toxic cultures.

Dr. J. C. Geiger collected, in February, 1920, at Klamath Falls 2 samples of soil and 3 specimens of bean stalks and beans (chicken outbreak 26). The soil, one bean and the bean stalks contained *B. botulinus*, type A.

Dr. A. A. Soule of Klamath Falls forwarded, in September, 1921, 15 samples of soils. The cultures prepared with these specimens gave the following results: Virgin soil (2), 2 *B. botulinus*, type A.

Garden soil (3), city pest house (1), 4 *B. botulinus*, type A.

Surface soil and swampy soil (2), weak toxins.

Garden soil (1), road soil (1), tule soil (1), tannery slough soil (1), surface dirt (1), spring yard (2), all negative.

Dr. H. J. Sears, Department of Bacteriology, University of Oregon Medical School, Portland, collected 2 samples of virgin, 3 of cultivated and 1 of non-fertilized soil (chicken yard) from the vicinity of Markham Hill. The cultures prepared with these specimens were nontoxic.

Summary: 4 virgin soils, 2 type A; 19 garden soils, barnyards, swamp soil, etc., 6 type A and 4 weak toxins; 12 fruits and vegetables, 5 type A and 7 weak toxins.

Total: 38 samples with 13 (24), or 34.2 (57.8) %, positive cultures.

Pennsylvania.—Two human outbreaks, one at Pittsburgh, due to home canned corn and one at Greensburg, caused by pickled olives packed in California.

Dr. George H. Hart, University of Pennsylvania Extension School, Philadelphia, forwarded, in April, 1921, 6 samples of soil (corral, corn field, poultry yard, pig pen, pasture, rye field) and 2 specimens of corn husks and beets. The following cultural results were obtained:

Soil from corn and rye field, 2 *B. botulinus*, type B.

Soil from field, pasture for 50 years, 1 *B. botulinus*, type A.

Soil from corral, 1 weak toxin.

In July, 1921, an additional 6 samples of moldy hay and corn from Montgomery and Chester counties, as well as moldy silage and hay were sent by Dr. Hart. Two specimens of silage were received from Pittsburgh, in May, 1921. The following cultures were toxic:

Sample of moldy hay, Chester County, *B. botulinus*, type B.

Sample of moldy corn fodder, *B. botulinus*, type A.

Summary: 6 manured or cultivated soils, 2 type B, 1 type A, 1 weak toxin; 10 feeds, silage, moldy hay, 1 type B, 1 type A.

Total: 16 samples with 5 (6), or 35.0 (37.5) %, positive cultures.

Rhode Island.—Dr. H. G. May, Agricultural Experiment Station of Rhode Island State College, Kingston, forwarded, in August, 1921, 6 samples of soil (rabbit pen, chicken yard, grain field, corn field, cattle pen and pasture) and 18 specimens of vegetables and feeds: silage, turnips (2), straw (2), dried beans, hay, beans (2), clover, beets (2), lettuce (2), spinach, peas, carrots and beet tops. The cultures prepared with these specimens proved nontoxic. One culture of spinach and soil of a corn field contained *B. tetani*.

Summary: 6 soils (cultivated or manured), all negative; 18 vegetables and feeds, all negative.

Total: 24 samples free from spores of *B. botulinus*.

South Carolina.—Dr. W. B. Aull, Associate Professor of Bacteriology, the Clemson Agricultural College, Clemson College, forwarded, in August, 1921, 7 samples of soil (hog pen, corn field, cow corral, grain field, poultry yard, clover field, cow pasture) and 15 specimens of vegetables and feeds (decayed leaves [2], beet tops, carrots [2], bean pods and stalks, beets, tops of kale, moldy hay [2], lettuce, pieces of corn stalks, tops of turnips [3]). The following cultures were toxic:

Beet and carrot tops, bean pods and stalks, 3 *B. botulinus*, type B.

Soil from grain field, 1 *B. botulinus*, type B.

Turnips, 1 weak toxin.

Summary: 7 soils manured or cultivated, 1 type B; 15 vegetables and feeds, 3 type B, 1 weak toxin.

Total: 22 samples with 4 (5), or 18.1 (22.7) %, positive cultures.

South Dakota.—Mr. C. D. Geidel, Director of the Minnesota Inspection Service, National Canners Association, St. Paul, forwarded, in January, 1921, 2 samples of soil and 2 specimens of corn husks procured from the Bigstone City Canning Company, Bigstone City. The cultures prepared with these specimens were nontoxic.

Dr. T. B. Taylor, Animal Health Laboratory, South Dakota State College, Brookings, forwarded, in April, 1921, 7 samples of soil (corn field [2], clover field, pig pen, cattle corral, pasture and poultry yard) and 6 specimens of roots (2), corn stalks, moldy hay and decaying vegetables (2). The cultures prepared with these were all nontoxic.

Summary: 9 soils cultivated or manured, negative; 18 vegetables and feeds, negative.

Total: 27 samples entirely free from spores of *B. botulinus*.

Tennessee.—One human outbreak in Memphis, due to ripe olives packed in California.

Dr. Maurice Mulvania, Department of Bacteriology, University of Tennessee, Knoxville, forwarded, in May, 1921, 7 samples of soil (corn field, cattle pasture, grain field, clover field, cattle pen, hog pen, poultry yard) and 13 specimens of vegetables and feeds (corn stalks, bean stover, bean stalk, pea vines, parsley, mustard, Irish potatoes, lettuce, beet stalk, young cabbage plant, straw [3]). The following cultures were toxic:

Irish potatoes, bean stalks, beet stalks, 3 *B. botulinus*, type A.

Soil from clover field, lettuce, mustard and decayed vegetation, 3 weak toxins.

Summary: 7 soils, cultivated or manured, 1 weak toxin; 13 vegetables and feeds, 3 type A, 2 weak toxins.

Total: 20 samples with 3 (6), or 15.0 (30.0) %, positive cultures.

Texas.—One human outbreak, due to sausages prepared in El Paso.

Mr. E. B. Reynolds, Division of Agronomy, Texas Agricultural Experiment Station, College Station, forwarded, in May, 1921, 7 samples of soil (cattle

corral, pig pen, poultry yard, corn, grain, clover and pasture fields) and 14 specimens of vegetables and feeds (corn stalks, decaying vegetation [2], moldy hay [2], bean pods and stalks, peas, lettuce, beet tops, turnip greens [2], beets, radishes and parsley). The cultures were all nontoxic.

Summary: 7 soils, cultivated or manured, all negative; 14 vegetables and feeds, all negative.

Total: 21 samples entirely free from spores of *B. botulinus*.

Utah.—Dr. T. B. Beatty, State Health Commissioner, Utah State Board of Health, Salt Lake City, forwarded, in December, 1920, 2 samples of garden soil and 2 of unbroken sage brush, surface soil from the vicinity of Salt Lake City. One sample of garden soil contained *B. botulinus*, type A.

Vermont.—Professor B. F. Lutman, Department of Botany, University of Vermont, Burlington, collected and forwarded, in May, 1921, 9 samples of soil (pasture land, pig pen, cattle yard, corn field, chicken pen, forest humus, clover field, pig yard, barley field) and 13 specimens of vegetables, feeds, etc. (chaff [2], moldy silage, forest leaves, corn stalks, radishes, decaying weeds from compost heap, straw, bean pods, potatoes, beets, bean stalks and droppings from hen roost). The following cultures were toxic:

Decaying weeds from compost heap, 1 *B. botulinus*, type B.

Soil from pig yard, 1 *B. botulinus*, type B.

Summary: 9 soils cultivated or manured, type B; 13 vegetables, feeds and decaying vegetation, type B.

Total: 22 samples with 2, or 9.0%, positive cultures.

Washington.—Fourteen human and 23 fowl outbreaks; one human outbreak each at Seattle, due to home canned string beans and asparagus; one at McKenna, due to home canned spinach, 6 at Yakima, due to home canned corn, one at Yakima, due to home canned asparagus, one at Toppenish, due to home canned spinach, one at Yakima, due to commercially packed milk, one at Walla Walla, due to home canned asparagus and one at Monroe, due to commercially packed ripe olives. One chicken outbreak at Yelm, due to home canned string beans, 4 at Yakima, due to home canned corn and 2 due to home canned peas and 16 due to buried raw potatoes.

Mr. A. W. Hansen, Chief, Seattle Station, Bureau of Chemistry, U. S. Department of Agriculture, Seattle, forwarded, in January, 1921, 6 samples of virgin soil taken at Roosevelt Heights, just north of Seattle, 10 samples of commercial truck garden soil from South Park, sample of manure and 4 specimens of vegetables (potatoes, onions, carrots and turnips). The cultures prepared with these specimens were all nontoxic.

Dr. C. R. Fellers, Pacific Fisheries Investigation, National Canners Association, Seattle, collected and forwarded, in February, 1921, from the vicinity of the University grounds 11 samples of soil (garden, lumber yard, beet garden, subsoil, mud and silt, sewer opening, virgin, marsh soil, flower beds, drug plant garden [2]) and 1 sample of manure. The specimen of mud and silt produced a weakly toxic culture.

Dr. J. C. Geiger collected, in May, 1921, near Toppenish, Yakima Valley, 34 samples of garden soil (spinach garden [10], garden [18], irrigation ditches [4] and field soil [2]). The cultures gave the following results:

Spinach garden, 1 *B. botulinus*, type A, 2 type B, 1 weak toxin.

Vegetable gardens, 3 *B. botulinus*, type A.

Similar samples were collected at Tieton, Yakima Valley: 10 samples of ranch soil and 4 specimens of soil and potatoes from pits suspected of con-

taining feed which caused avian botulism. The specimens developed toxic cultures, as follows:

Ranch soil, 3 *B. botulinus*, type A, 2 type B, 1 weak toxin.

Potato pits, 2 *B. botulinus* type A, 1 type B.

Additional samples were collected at random in the Yakima Valley: 6 samples of virgin soil, 6 of garden soil and 3 of pea garden soil. The following specimens developed toxic cultures:

Virgin soil, 5 *B. botulinus*, type A.

Garden soil, 1 *B. botulinus*, type A, 1 weak toxin.

Dr. J. C. Geiger collected, in July, 1921, near Yelm, 37 specimens of soil, manure, home canned peas and beans, etc. The following specimens furnished toxic cultures:

Eighteen soils, hen house (1), bean garden (6), cultivated (5), wheat field (3), pasture (1), garden (2), 17 *B. botulinus*, type A; 4 soil from irrigation ditches, 4 *B. botulinus*, type A; 3 virgin soils, 2 *B. botulinus*, type A, 1 weak toxin; 6 manures (hogs 3, cattle 3), 3 *B. botulinus*, type A, 1 weak toxin; 3 soil from house excavation, 3 *B. botulinus*, type A; 3 home canned peas (2), beans (1), 1 peas, 1 *B. botulinus*, type A.

In September, 1921, Dr. J. C. Geiger collected 20 samples of virgin soil along the eastern ledge of the Nisqually Glacier, Paradise Park, of the Mount Rainier National Park (elevation 5,000 to 6,000 feet); 7 cultures contained *B. botulinus*, type A, one type B and 5 gave weak toxins.

Summary: 36 virgin soils, 14 type A, 1 type B, 6 weak toxins; 41.6 or (58.3) %, positive; 98 garden, or irrigated soils, 32 type A, 4 type B, 4 weak toxins; 36.7 (40.8) % positive; 8 manure, 3 type A, 2 weak toxins; 8 vegetables and potatoes, 2 type A, 1 type B; 3 home canned peas or beans, 1 type A.

Total: 153 samples with 58 (70), or 37.8 (45.7) %, positive cultures.

West Virginia.—One human outbreak at Parkersburg due to pumpkin packed in Ohio.

Mr. Francis M. Morgan, Department of Soils, College of Agriculture and Agricultural Experiment Station, West Virginia University, Morgantown, collected and forwarded, in April, 1921, 8 samples of soils (poultry yard, cattle yard, horse pasture, clover, corn and wheat fields, pig pen, cold frames green house) and 12 specimens of moldy straw, corn stalks, decayed oat straw, cabbage heads, green peas, green beans, humus from compost heap, carrots, cucumber, radishes and manures from pig pen and horse stable.

One sample of soil from a field used for corn last year contained *B. botulinus*, type B.

Dr. J. C. Geiger collected 21 samples of virgin soil from the Alleghany Mountains in West Virginia.

One sample contained *B. botulinus*, type B, and one *B. tetani*.

Summary: 21 virgin soils, 1 type B; 8 manured and fertilized soils, 1 type B; 12 vegetables and manure, negative.

Total: 41 samples with 2, or 4.8%, positive cultures.

Wisconsin.—One human outbreak, due to commercially prepared sausages, at Milwaukee.

Mr. H. C. Kitchen, Director of Wisconsin Inspection Service, National Cannery Association, Madison, forwarded, in December, 1920, 2 samples of soil and corn husk each from Columbus, 1 sample of pea vines and soil each, Cedar Grove, Thiensville and Bloomer. Ten cultures were prepared. One soil sample from Cedar Grove, Sheboygan County, produced a weakly toxic culture.

Dr. J. C. Geiger collected, in October, 1921, 19 samples of virgin soil with pine decay from wooded land, vicinity of Milwaukee, and 9 samples from a celery farm of the same region.

One sample of soil from wooded, virgin soil contained *B. botulinus*, type B, and one other produced a weak toxin.

One sample of soil collected from a celery farm gave a weak toxin, while three others contained *B. tetani*.

Summary: 14 cultivated and manured soils, 1 weak toxin; 17 virgin soil, 1 type B, 1 weak toxin; 5 vegetable remnants, negative.

Total: 36 samples with 1 (3), or 2.7 (8.3) %, positive cultures.

Wyoming.—One human outbreak at Cheyenne, due to home canned corn.

Dr. Cecil Elder, College of Agriculture and Mechanical Arts, University of Wyoming, Laramie, collected and forwarded, in April, 1921, 12 samples of soil [cattle corrals (6), pasture (2), hog pen (1), poultry yard, grain field and clover field] and 10 specimens of feeds and vegetables [moldy hay (5), moldy barley, pea pods and stalks, parsnips, carrots and potato].

The specimen of moldy barley contained *B. botulinus*, type A. The soil samples from the poultry yard, cattle corral, Ranch (1) and potatoes produced weak toxins.

In June, 1921, one of the writers (K. F. M.) collected 30 soil samples in the Yellowstone National Park. The cultures prepared gave the following results:

Forest soil near Old Faithful (2), 1 type A; upper Geyser Basin (9), rock-slide near Butterfly Geyser (1), all negative; road embankment near Castle Geyser (1), 1 type A; Forest soil near Saw Mill Geyser (2), 1 type A; Blacksand Basin (forest 2, swampy meadow 1), all negative.

Vicinity of Yellowstone Lake (8): Lake sand, near Hotel (1), negative; soil of damp meadow near Camp (1), negative; fresh soil cut near road side, Camp (1), 1 type A; soil on tree roots (2), all negative; soil from horse corral (1), 1 type A; soil from Stevenson Island (2), 2 type A.

Grand Canyon of the Yellowstone (8): Red volcanic rock near Lower Falls (1), street cut near Lookout Point (1), Terrace near Lower Falls (1), Meadow near hotel (1), sidewalk Lower Falls (1), all negative; damp soil under glacial boulder near Canyon (1), 1 type B; summit of Mt. Washburn, damp soil (2), 2 type A.

Mammoth Hot Springs (5): Soil, squirrel hole on meadow (1), forest soil near Angel Trail (1), Devil's Kitchen (1), all negative; garden soil near hotel (1), 1 type A; soil near Boiling River (1), 1 type B.

Summary: 28 virgin soil (Yellowstone Park), 8 type A, 2 type B; 14 cultivated manured or garden soil, 2 type A, 2 weak toxins; 10 vegetables and feeds, 1 type A, 1 weak toxin.

Total: 52 samples with 13 (15), or 25.0 (38.8) %, positive cultures.

The statistical data presented in the preceding pages are summarized in table 1.

A brief analysis shows that 1,538 specimens, exclusive of California, have been studied; 375, or 24.3%, of the samples produced toxic cultures, although only 256, or 16.5%, could be identified by an antitoxin neutralization test. These figures are slightly changed by adding the findings reported for California, namely, 26.8%, or about one-quarter, of the field specimens collected in the United States contained spores,

which produced a *B. botulinus*-like poison in mass cultures. In 18.2% of the cultures, the presence of *B. botulinus* was definitely established by antitoxin tests.

TABLE 1
RESULTS OF EXPERIMENTS WITH SPECIMENS FROM VARIOUS STATES

State	Total Number of Spec- imens Exam- ined	Total Number of Toxic Cul- tures	Total Number of Typed Cul- tures	Type A	Type B	Types A and B	Un- typed	Per- centage Total Toxic Cul- tures	Per- centage Typed Cul- tures
Alabama.....	16	1	1	1	—	—	—	6.2	6.2
Arizona.....	1	—	—	—	—	—	—	0	0
Arkansas.....	41	6	1	—	1	—	5	14.6	2.4
(California).....	(624)	(206)	(139)	(100)	(35)	(4)	(67)	33.0	22.2
Colorado.....	69	17	12	12	—	—	5	24.6	17.4
Connecticut.....	24	6	1	1	—	—	5	25.0	4.1
Delaware.....	18	6	4	—	4	—	2	33.3	22.2
Florida.....	45	9	8	1	7	—	1	20.0	17.7
Georgia.....	20	5	5	—	5	—	—	25.0	25.0
Idaho.....	33	7	4	3	1	—	3	21.2	12.1
Illinois.....	39	5	2	—	2	—	3	12.8	5.1
Indiana.....	28	8	5	—	5	—	3	28.5	17.8
Iowa.....	34	3	1	1	—	—	2	8.6	2.9
Kansas.....	30	2	2	2	—	—	—	6.6	6.6
Kentucky.....	17	12	12	1	11	—	—	70.5	(70.5)
Louisiana.....	48	6	2	2	—	—	4	12.5	4.1
Maine.....	41	10	10	8	2	—	—	24.3	24.3
Maryland.....	30	17	15	1	12	2	2	56.6	50.0
Massachusetts.....	17	3	1	—	1	—	2	17.6	5.8
Michigan.....	120	10	5	1	4	—	5	8.3	4.1
Minnesota.....	46	5	2	2	—	—	3	10.8	4.3
Mississippi.....	19	6	3	—	3	—	3	31.5	15.7
Missouri.....	34	15	12	1	11	—	3	44.1	35.2
Montana.....	66	27	16	12	3	1	11	40.8	24.2
Nebraska.....	23	6	2	2	—	—	4	26.0	8.6
Nevada.....	25	11	9	9	—	—	2	44.0	36.0
New Hampshire.....	14	2	1	—	1	—	1	14.2	7.1
New Jersey.....	36	9	7	1	6	—	2	40.0	19.4
New Mexico.....	8	—	—	—	—	—	—	0	0
New York.....	31	8	6	6	—	—	2	25.8	19.3
North Carolina.....	20	6	—	—	—	—	6	30.0	0
North Dakota.....	14	3	1	1	—	—	2	21.4	7.1
Ohio.....	41	5	3	—	3	—	2	12.1	7.3
Oklahoma.....	24	4	1	—	1	—	3	16.6	4.1
Oregon.....	38	24	13	13	—	—	11	63.1	34.2
Pennsylvania.....	16	6	5	2	3	—	1	37.5	35.0
Rhode Island.....	24	—	—	—	—	—	—	0	0
South Carolina.....	22	5	4	—	4	—	1	22.7	18.1
South Dakota.....	17	—	—	—	—	—	—	0	0
Tennessee.....	20	6	3	3	—	—	3	30.0	15.0
Texas.....	21	—	—	—	—	—	—	0	0
Utah.....	4	1	1	1	—	—	—	25.0	25.0
Vermont.....	22	2	2	—	2	—	—	9.0	9.0
Washington.....	153	70	58	52	6	—	12	39.2	37.8
West Virginia.....	41	2	2	—	2	—	—	4.8	4.8
Wisconsin.....	36	3	1	—	1	—	2	8.3	2.7
Wyoming.....	52	16	13	11	2	—	3	30.7	25.0
Total (exclusive of California).....	1,538	375	256	150	103	3	119	24.3	16.5
Total (inclusive of California).....	2,162	581	395	250	138	7	186	26.8	18.2

One hundred and fifty, or 58.5%, of the typed cultures contained *B. botulinus*, type A; 103, or 40.3%, type B, and 3, or 1.2%, mixture of type A and B. The predominance of type A is probably due to the

findings made in the State of Washington, and is of little significance. In fact, it is not the relative numerical preponderance of one type over the other, but the geographical distribution of the various types which deserves special consideration in subsequent paragraphs. Attention is called to the negative findings made on the samples procured from New Mexico, South Dakota and Texas. It is quite likely that these results indicate an uneven distribution of the spores of *B. botulinus*, but it would be unwise to conclude from the limited data that the organism is not present in the soil of these states. The specimens collected in Arkansas, Connecticut, Illinois, Indiana, Louisiana, Michigan, Minnesota, Mississippi, North Carolina, North Dakota, Oklahoma, South Carolina, Tennessee and Wisconsin furnished a relatively large percentage of cultures which were weakly toxic and could not be identified by antitoxin tests. It was also noted that the typed cultures prepared with the samples obtained from these states contained only 1 to 5 minimum lethal subcutaneous guinea-pig doses per 2 c.c. Previous studies have shown that specimens collected in California, Oregon, Washington, Montana, and other states, yield, as 25 gm. samples during 10 days' incubation in peptic digest broth, toxic cultures which are fatal to guinea-pigs in dilutions of 1:1,000 to 1:10,000. Not infrequently, *B. botulinus* has been isolated in a pure state from these cultures, while innumerable attempts made with the specimens from the first named states, in which no strongly toxic cultures were found, have regularly been unsuccessful. Laboratory tests reported in a former paper indicate that a few spores added to soils produce, as a rule, weakly or nontoxic cultures. It is naturally possible that a number of weakly toxic cultures are the result of the recently described anaerobe isolated by Bengtson from the larvae of *Lucilia Caesar*, although repeated transplants and cultures of new samples of soil which originally gave weakly toxic cultures furnished in a few instances highly potent enrichments containing the toxin of *B. botulinus*. One is therefore forced to conclude that the field specimens collected in the 14 states mentioned harbored relatively few spores of *B. botulinus*. The practical significance of this observation will be considered in greater detail in the following.

The data presented in table 1 suggest a number of interesting points, which will be treated under the following headings:

- (1) The relative distribution of *B. botulinus* in different states of the United States.

- (2) The distribution of the two types of *B. botulinus*.
- (3) The telluric distribution of the spores.
- (4) The relation of the distribution of *B. botulinus* in the soil and its products to outbreaks of human and animal botulism.

(1) *The Relative Distribution of B. botulinus in Different States of the United States*.—With the exception of Virginia, every state of the Union supplied from 1 to 624 samples of soil and its products. Nevertheless, the survey cannot be considered in any way complete or exhaustive. Such a study is obviously very difficult and would consume an enormous financial outlay and several years of continuous work. A scrutiny of table 1 indicates that a number of states provided a small number of specimens which necessarily represent confined areas of the state. These facts should be fully appreciated and the deductions which will be made from the 1,538 cultural findings should be considered tentative, although they are in many ways suggestive.

For the sake of clearness and brevity, the figures dealing with the percentage of toxic and properly identified cultures are presented on chart 1. Four contrasting shades representing the percentages of 1 to 10 (1), 10 to 20 (2), 20 to 30 (3), and 30 to 70 (4), have been chosen for graphic illustration of the distribution in the different states. A rapid glance at chart 1 reveals three interesting facts, namely:

(1) The spores of *B. botulinus* are relatively rare in the samples of soil, etc., collected from the Middle States, the Great Plains States and those bordering the Great Lakes and the Mississippi River with its tributaries, the Missouri, Arkansas and Red, Des Moines and Illinois Rivers.

(2) The Atlantic States supplied specimens which produced frequently from 10 to 30% toxic cultures.

(3) The soil of the Pacific Coast and Rocky Mountain States was heavily infected with *B. botulinus*.

One state of the 14, bordering on the Mississippi River, namely, South Dakota, supplied nontoxic cultures, while Kentucky furnished 70.5%, Mississippi and Tennessee 15% and Missouri 35.2% toxic cultures. In Kentucky and in Missouri, collections were made in the vicinity of experiment stations. As far as Kentucky is concerned, it is known that for a number of years work on animal botulism has been carried out at the experiment station, and it is not unlikely that the percentage of positive specimens can be attributed to contamination of the premises with spores. The samples from Tennessee originated

in the neighborhood of Knoxville, or in close proximity to the Appalachian Mountains. Excluding these 4 states from consideration, it is clear that the soil of the Mississippi Basin contains the spores of *B. botulinus* in exceptional instances only. In fact, 9 states which are represented in this study by 311 specimens or one-fifth of all the samples tested in the United States, exclusive of California, gave less than 4.5% positive cultures. Sixty-four per cent. of these toxic cultures were weakly toxic and were probably the result of a very few heat resistant, viable spores.

The states of Michigan, Indiana, Ohio, possibly Pennsylvania and West Virginia, form a part of a region which has a low *B. botulinus* spore index. This statement is particularly well supported by the

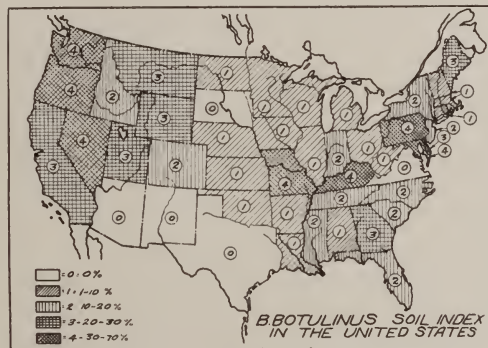


Chart 1

findings made on 120 specimens collected in Michigan; 62 soil samples derived from cultivated fields gave 3, or 4.8%, toxic cultures, while 10 virgin samples proved on repeated tests to be free from *B. botulinus*. Ohio and West Virginia gave 7.1 and 4.8% toxic cultures. One locality in Indiana supplied 17.8% positive samples, which contained the toxicogenic anaerobe, but judging from the number of additional negative specimens, this state can well be included in the group mentioned. It is regrettable that most of the material on which the data for Pennsylvania are based were collected in the eastern section of the state, and no information relative to the frequency of *B. botulinus* in the western part along the Alleghany and Ohio Rivers is available. Notwithstanding this fact, it appears that the valley of the Ohio River shares the same low percentage of *B. botulinus* in the soil as the Mississippi Basin and the lower portion of its tributary, the Missouri. The

15 states which furnished less than 10% positive cultures belong topographically to the Great Plains. The average altitudes of these states from which samples have been collected are between 1,000 to 2,000 ft. It is impossible to connect the limited data thus far collected with any geological formation. Most of the soil samples belong to the class of residual rock. Future studies must furnish the solution of the many questions which these observations suggest, but it is believed that the low *B. botulinus* index in the Great Plains and the Mississippi Valley is, in the light of the observations made in California, mainly governed by the absence of heavily infected virgin mountain areas.

Six of the 15 North and South Atlantic States supplied soil and vegetables which contained *B. botulinus* in from 20 to 50% of the cultures, while those derived from 4 states, namely, Vermont, New Hampshire, Massachusetts and Connecticut, were positive in less than 10%, usually 5%, of the tests. These two extremes are balanced by the findings in the states of Florida, North and South Carolina, New Jersey and New York, which furnished between 10 to 20% typical cultures. As a whole, 15 Atlantic States, represented by 421 specimens, gave 96, or 22.8%, toxic and 71, or 16.9%, typable cultures, which were definitely proved to contain *B. botulinus*. This organism is, therefore, somewhat more frequently encountered in the Atlantic States than in those of the Great Plains and the Mississippi Valley. Pennsylvania and Maryland are exceptions, but it is not unlikely that the high percentage of positive cultures is the result of chance sampling or is dependent on the same soil stratum in both states, namely, a part of the Blue Ridge Mountain soil which supplied the samples. The relatively frequent findings of *B. botulinus* spores in the draining areas of these mountains, namely, New Jersey, Delaware and Maryland, lends considerable support to the last mentioned view and suggests the Blue Ridge Mountains as a breeding place for the neighboring valleys and river beds.

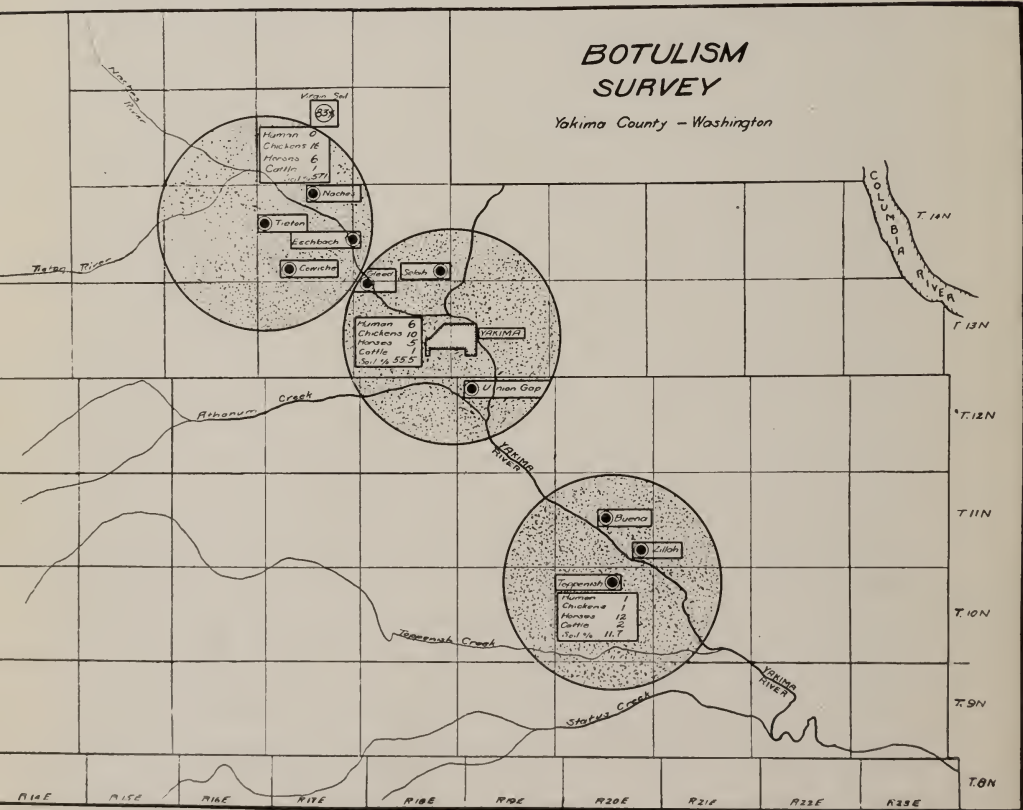
The number of examinations made on material supplied from the 4 North Atlantic States which gave less than 10% positive cultures is too small to justify any definite conclusions. The states are surrounded on both sides by soil strata which harbor *B. botulinus* spores in a fairly large percentage of specimens. Attention is called in this connection to the existence of the organism in virgin forest soil in Main and New York. It is therefore not unlikely that extended sampling in Vermont, New Hampshire, Massachusetts and Connecticut may radically change the aspect of the data thus far collected.

The soil and vegetable specimens obtained from the South Atlantic States, Georgia, North and South Carolina, originated in the interior, while those of Florida represent coast, as well as inland, collections. Georgia and South Carolina yielded 25 and 18.1% positive cultures; soil strata of the foothills of the Blue Ridge Mountains supplied the samples. In Florida, the differences between the coast and the interior are clearly shown by the absence of *B. botulinus* in the samples collected around Miami and the fairly high percentage of infected material procured from the neighborhood of Gainesville. This interesting comparison indicates that virgin soil on the Atlantic Coast may be free from the spores of *B. botulinus* and, furthermore, emphasizes the conclusion previously drawn that the findings made on one topographical section do not necessarily apply to the state as a whole.

The results reported for the Middle and Atlantic States are in striking contrast to those revealed by the specimens procured from the Western States. Ten states, exclusive of California, supplied 449 samples, with 126, or 28%, toxic and properly identified cultures. Topographically these states form a part of the Cordilleran system, and, with the exception of Colorado and Idaho, furnish a *B. botulinus* soil index of 20%, while in 3 states, Oregon, Nevada and Washington, it is more than 30%. In the last named state, every section was found to contain this anaerobe, and the detailed findings made in the Yakima Valley deserve brief consideration. The percentage figures shown in chart 2 suggest that *B. botulinus* is, as in California, an inhabitant of the virgin soil. It may be mere coincidence due to methods of sampling, but the data indicate that from the mountain ranges toward the valleys, following the descending course of the rivers, there is a progressive reduction in the infection index of the soil. For example, virgin mountain soil contained *B. botulinus* spores in 83% of instances, while earth collected in the adjacent Tieton and Yakima Valley sections gave 57.1 and 55.5% positive cultures. On the other hand, the Toppenish area, which is farther removed from the source of the Yakima River and its tributaries, supplied comparatively few infected soil specimens (11.7%). The soil is equally polluted in the vicinity of Yelm and the virgin soil along the eastern ledge of the Nisqually Glacier on Mount Rainier.

The demonstration and isolation of *B. botulinus* from virgin sage brush soil and uninhabited sections of Nevada forcibly support the conclusion that this organism is universally distributed in the Western States. Additional evidence was also secured by carefully sampling

virgin districts in 3 National Parks: Yellowstone, Glacier and Mount Rainier, as well as the region surrounding Pike's Peak. Twenty-six to 40% of the soil samples collected in these regions yielded positive cultures and frequent isolations of the anaerobe. *B. botulinus* has been located at altitudes around 11,000 ft. in soil layers of glacial origin and on an isolated island of the Yellowstone Lake and has, by repeated



tests, been demonstrated in pure cultures. This evidence strongly refutes the conception of an intestinal habitat of the organism. Unfortunately little or no attention was paid to the geological structures which furnished the positive virgin soil specimens, and this survey does not permit an answer to the question: Can *B. botulinus* breed in these regions, and what are its biological functions? Work to solve these mysteries is in progress and will be reported when available.

Based on this survey, it must be concluded that *B. botulinus* is a common soil anaerobe of the Western States or the Cordilleran system; it is less frequently encountered in the Atlantic States and relatively rare in the Middle States, Great Plains and the Mississippi Valley.

(2) *The Distribution of the Two Types of B. botulinus.*—Through the early studies of Leuchs² and the recent work of Burke,³ it is known that the antitoxin prepared against types A and B toxins is specific for the homologous toxin and will not protect against the heterologous one. This fact has been used to identify the toxin generated in the cultures prepared with the field specimens. A total of 256 cultures has been typed or identified by the isolation of *B. botulinus* in pure culture. It was recognized that certain states furnished only one type of *B.*

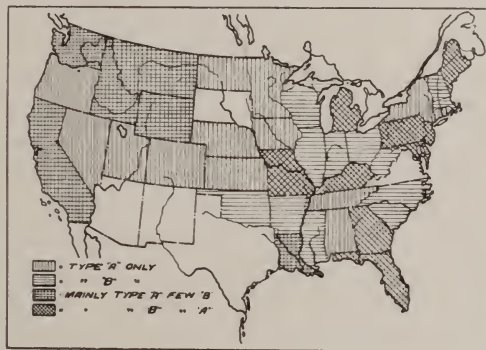


Chart 3

botulinus, namely, type A or B, while some gave, for example, predominantly type A, with one or two cultures yielding type B toxin, and vice versa. By grouping these data according to states and by using 4 contrasting shades, chart 3 has been obtained.

It is quite evident that the distribution of the 2 serologic types of *B. botulinus* divides the United States roughly into 2 distinct areas: The Western States, inclusive of the Great Plains, and the Mississippi Valley together with the Atlantic States. In a general way, the soil area spreading from the 95 degrees longitude westward to the 125 degrees longitude harbors mainly type A, while that extending from 95 degrees longitude eastward to the 65 degrees longitude supplies *B. botulinus* type B. The Mississippi Valley, with the valleys of its

² Ztschr. f. Hyg. u. Infektionskr., 1910, 65, p. 55.

³ Jour. Bacteriol., 1919, 4, p. 555.

tributaries, the Ohio, the Red and a part of the Missouri, as well as the Great Lakes region, is characterized by a striking predominance of type B. Similarly prevalent is this type in the Atlantic States of Maryland, Delaware, New Jersey, Georgia, South Carolina, etc., while the scattered findings of type A, in Maine, New York and Pennsylvania indicate the probable existence of seed beds in the form of virgin forests and mountains. Alabama, Connecticut and Tennessee supplied only type A toxins, but it is not unlikely that more extensive sampling would also place these 2 states in the type B. area.

For several years, it was known that type A was prevalent in the Western States, and the data illustrated by chart 3 fully confirm the statement made in a former paper that this type is primarily an inhabitant of the virgin soil from which it may be scattered by the streams and rivers to distant regions, even invading the Missouri and Mississippi Valleys (see Missouri and Louisiana). In Colorado, Oregon, Nevada, Utah, North Dakota, Nebraska, etc., type A is the only type encountered, while in Washington, Idaho, Montana and Wyoming type B is occasionally found; in every instance, the specimens infected with the spores of the latter type were either soil or vegetables from gardens or forest or moraine soil rich in decayed plant material. One sample of moldy alfalfa hay from Bozeman, Mont., contained a mixture of type A and B. The facts so revealed agree perfectly with the data presented in a former paper, namely: Type B is probably an adaptation mutant to the physical and chemical influences encountered by type A in tilled and cultivated soil. Whether the same explanation applies to the conditions in the Middle and Atlantic States cannot be stated with certainty. It may be mere coincidence, but by comparing chart 3 with a recent map showing the density of population in the United States according to the 1920 census, it will be noted that the type B area is nearly covered by that holding a population of from 18 to 400 persons per square mile. In other words, the soils which are subjected to intensive cultivation and fertilization contain, as a rule, *B. botulinus*, type B. A similar condition apparently exists in Canada and on the European continent.

Further comparison of chart 3 with chart 1 indicates that the specimens derived from states which yielded only, or at least predominantly, type B, produced also a low percentage of toxic cultures. When type B is isolated, the number of spores in the soil and its products is relatively small. This interesting condition was first noted in California and has since then been confirmed by the studies made in other states of the

Union. These facts have an encouraging aspect from the standpoint of botulism prevention, namely, intensive agriculture may in generations suppress *B. botulinus* as a soil anaerobe and consequently eliminate the danger of spore contamination on vegetables, fruits and feeds, provided the rivers and streams draining heavily infected areas of type A cease to reinfect these districts. Extensive irrigation as practiced in California and Colorado has been and is probably to a great extent responsible for the pollution of the cultivated fields and orchards with highly heat resistant spores of *B. botulinus*, by nature only present in the mountain soil. The significance of type B in its relation to human botulism is discussed in a subsequent paragraph.

(3) *The Telluric Distribution of B. botulinus.*—In chart 4, the cultural results of the 1,538 field specimens are arranged in graphic form according to their telluric origin, namely, virgin, cultivated, garden and pasture soil, dirt from animal corrals and vegetables and feeds. This presentation emphasizes the predominance of *B. botulinus* in virgin and pasture soil and the relatively low percentage of positive cultures in soil and manure collected from animal corrals, pig pens and chicken houses. As a whole, the data correspond closely to those already mentioned for California. Earth contaminated with manure or animal excreta rarely carries the spores of *B. botulinus*. In fact, fertilization cannot be considered a factor contributing to the pollution of the soil. Moreover, these columns are responsible for the suggestions made in the preceding paragraphs, namely, cultivation and cropping of the soil reduces or dilutes the number of spores to such a degree that the methods employed for detection fail to demonstrate their presence.

This view is also supported by the examination of soil products. Soil stratum furnishing nontoxic or weakly toxic cultures frequently supplied vegetables or feeds, which contained *B. botulinus*, type B. It is possible that the concomitant anaerobes or aerobes of the cultivated or manured soil inhibit the growth or the toxin production of the few spores of *B. botulinus* present in the usual amounts chosen for these tests. As a rule, the vegetable samples examined have been less heavily polluted with various antagonistic soil bacteria and demonstration of the toxicogenic anaerobe has been more readily accomplished than with soil specimens. In any case, 1 gm. of cultivated or manured garden soil contains quantitatively fewer spores than the same amount of virgin soil.

The cultural findings made on vegetables and feeds reflect the flora of the soil and may indicate the potential danger from *B. botulinus*

in a community. In this connection, it should, however, be emphasized that the mere demonstration of the organism in feeds does not signify that this material has in the past or may in the future cause animal

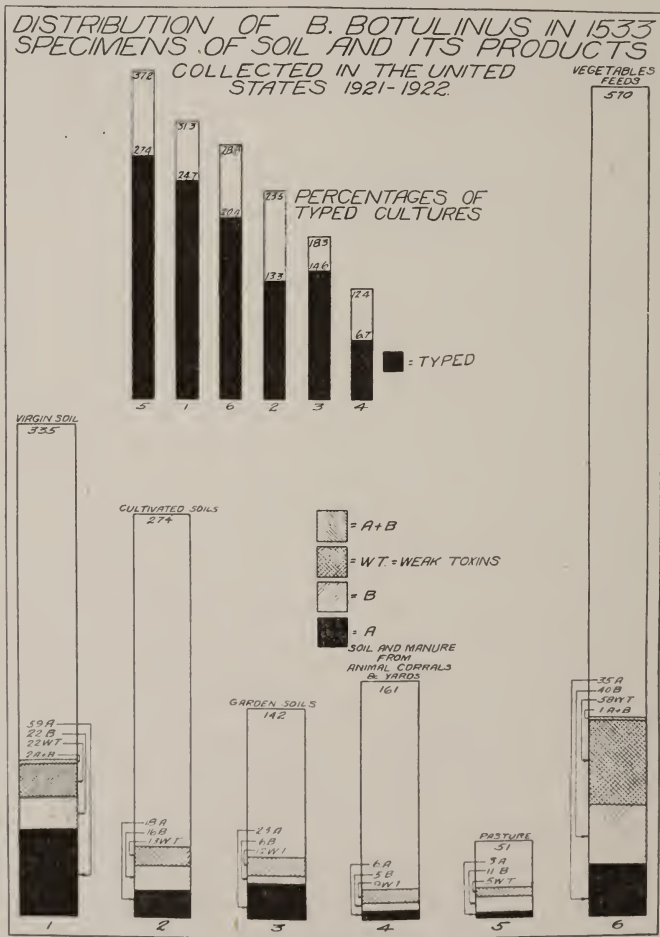


Chart 4

botulism. A number of workers dealing with certain animal diseases in the Middle Western States have repeatedly failed to appreciate these facts, and they have invariably made the diagnosis of botulism provided they were able to isolate *B. botulinus* from the suspected feed or the spleens and livers of dead horses, swine and sheep.

In table 2, the various vegetables, fruits and feeds which have been examined are tabulated and the percentages of positive cultures have been calculated for each important plant material. There is no doubt that every soil product may sometimes carry the spores of *B. botulinus*,

TABLE 2
PERCENTAGE OF POSITIVE CULTURES IN VARIOUS VEGETABLES, FRUITS AND FEEDS

Vegetables and Feeds	Total Number Examined	Number of Positive Cultures	Percent of Positive Cultures
Cornhusks, leaves and stalks.....	80	6	7.5
Peapods, stalks and leaves.....	51	3	5.4
Moldy hay.....	44	7	15.9
String beans, pods and stalks.....	44	14	32.7
Beets, roots and tops.....	37	6	16.2
Decayed vegetation.....	29	6	20.6
Tomato plant and roots.....	24	2	8.3
Lettuce.....	21	3	14.2
Carrots.....	18	2	11.1
Turnips.....	18	2	11.1
Cabbage.....	16	3	18.7
Ensilage.....	15	3	20.0
Radishes.....	15	1	6.6
Decayed straw.....	13	1	7.6
Straw.....	10	0
Bean roots.....	10	1	10.0
Spinach.....	10	0
Lima beans.....	9	1	11.1
Cucumber root.....	8	0
Pumpkin vines.....	8	0
Sweet potatoes.....	8	1	12.5
Swiss chard.....	8	0
Potatoes.....	8	2	25.0
Onions.....	8	1	12.5
Forage.....	6	2	33.3
Alfalfa.....	4	1	25.0
Parsnips.....	4	0
Asparagus.....	4	1	25.0
Oats.....	4	1	25.0
Parsley.....	4	0
Soya beans.....	4	1	25.0
Cherries.....	3	0
Dandelions.....	2	1
Hominy.....	2	0
Beet pulp.....	2	0
Mango tops.....	2	0
Red pepper.....	2	0
Mustard.....	2	0
Daffodils, cornfodder, wheat head, squash, crabapple, rutabaga, moldy barley, canna tops, cauliflower, curari roots, clover, strawberries (one each).....	13	3
Total 50 soil products.....	570	75	

but in the light of the epidemiology of botulism in this country, it is significant that string beans and leaves furnished the highest percentage of toxic and identified cultures. In this connection, it should be recalled that 45 instances are on record in which home or commercially packed string beans caused outbreaks of human (20) and avian (25) botulism. The factors responsible for the frequent contamination of string bean pods or vines deserve more careful experimental investigation, not

only from a biologic but also from a preventive standpoint. Plant material undergoing decay and fermentation, as for example vegetables, moldy hay and ensilage, supplied a high percentage of toxic cultures and isolations of *B. botulinus*. According to the reports accompanying the shipments of these samples, it was known that some of the moldy hay and ensilage containing *B. botulinus* had been consumed by horses and cattle with impunity. A few toxicologic examinations conducted in this laboratory failed to demonstrate the presence of *B. botulinus* toxins in these samples. These and similar observations cast serious doubt on the majority of claims which in recent years associate any mysterious animal disease with botulism. In fact, it is evident from the data presented in table 2 that *B. botulinus* is frequently found in moldy hay and ensilage but, so long as these feeds are free from toxin, they may be ingested by herbivorous animals without causing botulism.

From a biologic standpoint, it is by no means surprising to find *B. botulinus* frequently associated with decaying plant material. The organism, being a spore-bearer, rarely meets in nature the conditions suitable for its growth, and its continued existence would be impossible without some resting stage. In soil, conditions favorable to the growth probably occur when decaying vegetation furnishes the necessary food material. These conditions probably develop just often enough so that the spores do not diminish in number. This behavior deserves further study, but this possibility should be fully appreciated in canneries and utmost care exercised in the disposal of waste products. It is not unlikely that the most resistant spores of *B. botulinus* are not introduced into the packing plant on the fresh and sound vegetables and fruits, but are made available under unsanitary conditions of waste disposal. The principles which apply to the suppression of heat resistant thermophiles can unreservedly be used in the control of *B. botulinus* spores.

A total of 50 specimens of beans, cucumber and tomato roots, not tabulated, revealed only one positive culture, while the vines and leaves of the same plants from the same source furnished 10 or 20% positive cultures. These and similar tests should be made on a larger series of samples, but they confirm in a general way the previously made observations that *B. botulinus* occurs more often in the surface layers of the soil and consequently can cause dust borne contaminations of sound fruits on the trees. This condition has repeatedly been noted in the olive orchards of California.

TABLE 3

NUMBER OF OUTBREAKS OF BOTULISM COMPARED WITH PERCENTAGE FIGURES OF SOIL AND VEGETABLE SPECIMENS CONTAINING SPORES OF B. BOTULINUS

State	Botulism Outbreaks						Percent of Specimens Containing B. botulinus Spores			
	Human				Fowl: Locally Grown, Home Packed Food Products	Horse and Mules: Locally Grown Feed, etc.	Typed	Type A	Type B	Type A and B
	Total	Locally Grown, Com- mer- cially Packed	Locally Grown, Home Packed or Pre- served	Un- known						
Arizona.....	1	(1 Ohio)	?	22.2	78.4	22.1	3.1
California.....	47	11 (1 Ohio)	29	6	53
Colorado.....	3	1 (?)	1	..	3	(1)	17.4	100.0
Dist. Columbia..	1
Florida.....	1	..	1	17.7	12.5	87.5
Idaho.....	3	..	3	12.1	75.0	25.0
Illinois.....	1 (?)	1 (?)	1	1	5.1	100.0
Indiana.....	3	..	1	17.8	100.0
Iowa.....	1 (?)	(2 Cali- fornia)	2.9	100.0
Kansas.....	..	1 (?) (Colo- rado)	6.6	100.0
Kentucky.....	1	70.5	100.0
Maine.....	1	..	1	24.3	80.0	20.0
Massachusetts...	3	..	2	1	5.8	100.0
Michigan.....	3	0	4.1	20.0	80.0
Montana.....	1	(3 Cali- fornia) 0 (1 Cali- fornia)	24.2	75.0	18.7	6.3
Nevada.....	5	..	36.0	100.0
New Jersey.....	1	..	1	19.4	14.3	85.7
New York.....	5	0	3	1	19.3	100.0
Ohio.....	2	3 (Cali- fornia, Arizona, W. Vir- gina) (2 Cali- fornia)	7.3	100.0
Oregon.....	4	0	4	..	2	..	34.2	100.0
Pennsylvania...	2	0 (1 Cali- fornia)	1	35.0	40.0	60.0
Tennessee.....	1	0 (1 Cali- fornia)	15.0	100.0
Texas.....	1	1	0	0
Washington.....	14	1	12	1	23	..	37.8	89.5	11.4
West Virginia....	1	0 (1 Ohio)	4.8	100.0
Wisconsin.....	1	1	8.3	100.0
Wyoming.....	1	..	1	25.0	84.7	15.3
Total 27 states..	102	32	61	9	88	3				

(4) *Relation of Distribution of B. botulinus in the Soil and Its Products to Outbreaks of Human and Animal Botulism.*—In table 3, the number of botulism outbreaks thus far recognized in the different states is compared with the percentage figures of soil and vegetable specimens found to contain the spores of *B. botulinus*.

It is not surprising to note that when the percentage figures of positive cultures exceed 20 or 30%, and particularly when type A predominates, human and animal botulism is not infrequent. California, Colorado, Idaho, Maine, Nevada, New York, Oregon, Washington and Wyoming are excellent examples of these conditions. The states furnishing *B. botulinus*, type B either report no outbreaks, or botulism is caused by food products which have not been preserved by heat (sausages, hams, etc.). Comparative studies have shown that the spores of the majority of *B. botulinus*, type B strains are, as a rule, less heat resistant than those of type A (average of 23 strains type B, at 105 C. = 37 minutes and of 33 strains type A, at 105 C. = 62 minutes). It is, therefore, not unlikely that the absence of botulism in the states, which primarily harbor type B is due to the relative scarcity and possibly the low heat resistance of the prevailing spores of *B. botulinus*. This explanation is based on the strains thus far available, but it should be stated with utmost emphasis that spores of type B have been grown in this laboratory which have withstood a temperature of 105 C. for at least 65 minutes (strains 13, 41, 77 and 63) or the average resistance of type A strains.

No assurance can be given that such spores may not occasionally enter the food product to be preserved, and it would be most unwise to disregard in a Middle Western or Eastern state vigilance and rigid procedures of sterilization merely on account of the conditions indicated by this survey. From a practical standpoint, the spores of *B. botulinus* are ubiquitous; they may be found anywhere and any time, although certain locality variations deserve the attention of the biologist.

Outbreaks of fowl botulism are usually due to preserved vegetable products, and the same factors which lead to human cases are responsible for these fatalities. In fact, an approximate estimate of spoilage due to *B. botulinus* can be made from the various outbreaks of botulism among barnyard fowls, which developed symptoms of the disease or died following the consumption of poisonous, spoiled food. It is to be regretted, therefore, that reliable epidemiologic data relative to the number of outbreaks of botulism among fowls in states other than California and Washington are not available. On observation, in which

spoiled corn containing *B. botulinus* type B was the cause, has been reported from Washington, D. C. In another, which occurred in New Jersey, the type of the organism was not determined; while *B. botulinus* type A was found incidentally in a sample of chicken manure collected at East Lansing. By analogy with human outbreaks, it must be suspected that localities yielding a high percentage of *B. botulinus* in soil will furnish outbreaks of avian botulism. This statement deserves additional investigation and is made with reserve, as dependable data are not available. The same must be said with regard to the so-called outbreaks of equine, bovine and porcine botulism. In California, where every opportunity has been selected to verify the clinical diagnosis of equine botulism by careful laboratory tests, it has been impossible to find even one instance in which the suspected moldy hay, corn or straw contained the toxin of *B. botulinus*. The bacteriologic demonstration of *B. botulinus* in the tissues of horses and of cattle cannot be used as a criterion or as proof that certain forms of "forage poisoning" are actually botulism. Laboratory findings also indicate that this anaerobe may sometimes be demonstrated in the organs of animals which die of other diseases than botulism. Furthermore, states with a relatively low *B. botulinus* soil index report extensive outbreaks of "forage poisoning," and vice versa. In the light of these conditions, it is obviously impossible to draw deductions which can demonstrate any relationship between spore prevalence and equine or bovine botulism.

CONCLUSIONS

A general survey, during which 1,538 soil, vegetable, feed and manure specimens of every state of the United States, except Virginia, have been studied for the presence of the spores of *B. botulinus*, reveals the following facts:

1. *B. botulinus* is a common soil anaerobe of the Western States of the Cordilleran system. It is less frequently encountered in the Atlantic States and is relatively rare in the Middle States, the Great Plains and the Mississippi Valley.

2. The soil of the Western States, inclusive of the Great Plains, yield, mainly, *B. botulinus*, type A, while the Mississippi Valley and Great Lakes region is characterized by a striking predominance of type B. Similarly prevalent is this latter type in the Atlantic States of Maryland, Delaware, New Jersey, Georgia and South Carolina, while scattered findings of type A in Maine, New York and Pennsyl-

vania indicate the existence of breeding places in virgin forests and mountains. Soils which are subjected to intensive cultivation and fertilization contain, as a rule, *B. botulinus*, type B.

3. *B. botulinus* spores are far more prevalent in virgin and pasture soils than in dirt, soil or manure collected from animal corrals, pig pens, etc. Vegetables, fruits and feeds are frequently contaminated with the spores of *B. botulinus*. String bean pods and leaves, moldy hay, ensilage and decayed vegetation may yield a relatively high percentage of positive cultures.

4. Human and animal botulism is not infrequent in those states in which *B. botulinus*, type A, predominates, or in which the percentage figures of positive cultures exceeds 20 to 30%. From a practical standpoint, however, *B. botulinus* is ubiquitous, and this survey gives no assurance that heat resistant spores cannot be found anywhere and at any time.

5. The theory which claims that all the pathogenic anaerobes are regular inhabitants of the intestinal canal of animals deserves renewed investigation in the light of this survey on *B. botulinus*.

THE DISTRIBUTION OF THE SPORES OF *B. BOTULINUS* IN THE TERRITORY OF ALASKA AND THE DOMINION OF CANADA. V *

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In order to supplement the findings reported in the preceding papers, a limited number of soil specimens obtained from Alaska and the Dominion of Canada have been examined. This bacterio-geographic study serves to answer two questions: 1. Does *B. botulinus* occur in the soil of Alaska, and is the salmon canning industry endangered by this organism? 2. Can *B. botulinus* be demonstrated in the virgin forest and mountain soil of Canada?

No outbreaks of botulism due to commercially canned salmon have thus far been reported, probably on account of the fact that *B. botulinus* produces rapid and marked spoilage, which leads to an elimination of the abnormal tins before they reach the consumer. From a biologic and topographical standpoint, it was of interest to search for *B. botulinus* along the Alaskan river banks and Coast stations which house the various salmon canneries. Previous studies had shown that the rivers of the Pacific Coast slope serve as distributors of the spores from the mountains, which are the natural breeding places, so that the finding of *B. botulinus*, type A, in two sound clams removed from San Francisco Bay was not surprising. It was originally contemplated to study soil specimens collected at various sections of the Cordilleran system, extending along the Pacific Coast from the state of Washington to the Aleutian Islands. Unfortunately, for financial reasons, such a systematic survey was impossible, and the few samples which were procured from Alaska can only serve as guides for future investigations.

With the exception of one outbreak, reported by Glancy¹ and supposedly caused by government inspected meat or canned beets near Dawson City, Yukon, no human or animal botulism cases have been reported from Canadian territory. Samples of soil have been collected from various regions of the Dominion, and the results of the examinations form the basis for this communication.

Received for publication, July 26, 1922.

* This study was aided by grants from the National Cannery Association, the Cannery League of California and the California Olive Association.

¹ Canadian Med. Assn. Jour., 1920, 10, p. 1027; Report 82 in the Epidemiology of Botulism, Bulletin U. S. Public Health Service, in press.

EXPERIMENTAL DATA

The methods outlined in the preceding papers have been followed in the collection and examination of the specimens. The results are herewith briefly reported.

Alaska.—Dr. E. D. Clark, Director of Pacific Fisheries Investigations, National Cannery Association, Seattle, Wash., forwarded, in September, 1921, 7 samples of soil, which had been collected during the summer, at the following stations in Alaska: Alitak, Chignik River, Larsens Bay, Ikatan, Port Moller, Copper River (silt) and Copper River Bank.

The culture prepared with the samples collected at the Copper River Bank was toxic and killed 2 guinea-pigs with symptoms of botulism on the 5th and 8th day. The type could not be determined as the animals inoculated with serum invariably showed slight indefinite symptoms and recovered.

Canada.—In December, 1920, Dr. J. C. Geiger collected, in the vicinity of Vancouver, 6 samples of soil and 9 specimens of carrots, spinach, cabbage (2), greens and beets. Two samples of soils produced weak toxins.

In September, 1921, Dr. J. C. Geiger collected 20 samples of virgin soil (?) and 4 specimens of green moss along the tract of the Seymour Water supply near Vancouver, British Columbia. The cultures gave the following results:

Green moss samples, 3 *B. botulinus*, type A.

Virgin soil (?), 3 *B. botulinus*, type A, 2 weak toxins.

In October, 1921, Dr. J. C. Geiger collected 29 samples of virgin soil from the vicinity of Victoria and Lefroy Glacier, above Lake Louise, and at the foot of Mount White, Glacier, British Columbia. The cultures repeatedly gave the following results:

Red virgin soil (20), 4 *B. botulinus*, type A, 1 type B, 3 weak toxins.

Gray moraine and glacier soil (4), 2 *B. botulinus*, type B, 1 type A and B.

Black soil from Big Beehive (7,500 feet) (5), 1 *B. botulinus*, type A.

Dr. Henri Larouche, Chief Division of Laboratories, Montreal, submitted, in November, 1921, 16 samples of garden and road soil collected from the following counties in the Province of Quebec: St. Eustache (2); Chemin Ste. Rose-Pont Viau; Ste. Ursule, Co. Maskinonge; Pointe St. Charles, Montreal; St. Agnes de Dundee, Co. Huntingdon; Huntingdon; Bordeau; St. Cyr, Co. Richmond; Melbourne, Co. Richmond; St. Paul l'Ermite; L'Epiphanie; St. Leonard de Port Maurice; St. Michel; St. Jacques l'Achigan; Rawdon. The cultures were all nontoxic.

Dr. Frank T. Shutt, Dominion chemist, Central Experimental Station, Ottawa, kindly submitted, in December, 1921, 16 carefully collected and packed samples of virgin and manured soils procured from 8 provinces of Canada. The cultures furnished the following results:

Virgin soil, Charlottetown, Prince Edward Island, 1 *B. botulinus*, type A.

Manured soil, Napan, Nova Scotia, 1 *B. botulinus*, type A.

Virgin soil, Cape Rouge, Quebec, 1 *B. botulinus*, type A.

Virgin soil, Ottawa (C. Exp. Stat.), Ontario, 1 *B. botulinus*, type A.

Manured soil, Ottawa (C. Exp. Stat.), Ontario, 1 *B. botulinus*, type B.

The samples of manured soil, Charlottetown, Prince Edward Island; virgin and manured soil, Fredericton, New Brunswick; virgin soil, Nappan, Nova Scotia; manured soil, Cape Rouge, Quebec; virgin and manured soil, Brandon, Manitoba; Invermere, British Columbia, and Lethbridge, Alberta, were apparently free from spores of *B. botulinus*.

Summary: 61 virgin soil, 14 type A, 3 type B, 1 type A and B, 5 weak toxins; 30 cultivated or manured soil, 1 type A, 1 type B, 2 weak toxins; 9 vegetables, negative.

Total: 100 samples with 20 (27), or 20 (27.0) %, positive cultures.

The laboratory findings are summarized in table 1.

TABLE 1
SUMMARY OF LABORATORY FINDINGS

	Locality	Specimens	Total Number of Specimens Examined	Number of Toxic Cultures	Un-typed Cultures	Type A	Type B	Type A and B	Percentage of Positive Samples
192	ALASKA Various stations.....	Soils	7	1	1	—	—	—	—
92	CANADA Vancouver, B. C.	Soils (6): vegetables (9)	15	2	2	—	—	—	—
187	Vancouver, B. C.	Soils (20): green moss (4)	24	8	2	6	—	—	25.0
200	Glacier, Lake Louise, B. C.	Soils	29	12	3	5	3	1	31.0
213	Province of Quebec....	Soils	16	—	—	—	—	—	—
215	Charlottetown, P. E. I.	Virgin and manured soil	2	1	—	1	—	—	—
	Fredericton, N. B.	Virgin and manured soil	2	—	—	—	—	—	—
	Nappan, N. S.	Virgin and manured soil	2	1	—	1	—	—	—
	Cape Rouge, Quebec....	Virgin and manured soil	2	1	—	1	—	—	—
	Ottawa, Ont.	Virgin and manured soil	2	2	—	1	1	—	—
	Brandon, Man.	Virgin and manured soil	2	0	—	—	—	—	—
	Invermere, B. C.	Virgin and manured soil	2	0	—	—	—	—	—
	Lethbridge, Alberta....	Virgin and manured soil	2	0	—	—	—	—	—
	Total: Alaska.....		7	1	1	0	0	0	
	Canada.....		100	27	7	15	4	1	
			107	28	8	15	4	1	

It has been pointed out that the number of specimens secured from Alaska is insufficient to warrant any definite conclusions. The coast soil of the Aleutian Archipelago is apparently free from spores of *B. botulinus*. One specimen collected from the bank of the Copper River produced on 2 occasions weakly toxic cultures which could not be typed. This river drains the Nuzotin and Wrangel Mountains, or a

part of the Cordilleran system. *B. botulinus* may theoretically be expected in these soil stratum, and it is most unfortunate that only 2 specimens have been collected from this region. Future studies should consider the areas along the Copper and Yukon Rivers. Tests should be made, particularly in the mountains around Mt. Allen and Mt. Kimball. Until these examinations have been made, the question whether or not *B. botulinus* may play a rôle in the canning of Alaskan salmon cannot be answered.

Twenty-seven, or 27%, of the 100 specimens collected in Canada furnished toxic cultures. In 20 cultures, *B. botulinus*, type A, as well as Type B, were repeatedly demonstrated. This anaerobe is widely distributed in the Dominion, although the Western section, namely, British Columbia and particularly the Canadian Rockies, furnishes the highest percentage of positive cultures. Attention is called to the isolation of *B. botulinus*, type A and type B, from glacier and mountain soil around Lake Louise. These findings fully confirm those made in the Glacier National Park and support the conception that *B. botulinus* is a common soil anaerobe of the Cordilleran Mountain system. *B. botulinus*, type A, predominated, although type B occurred occasionally either alone or in conjunction with type A. The soil samples containing type B consisted in two instances of typical moraine sand with little decaying vegetation. Without additional geologic information, it was impossible to decide whether the spores had been washed into the soil stratum or whether the organism had actually developed in this earth material. Judging by previous findings on Mount Rainier and in the Glacier National Park, it is not unlikely that the first stated view is the correct one.

Three virgin soil specimens from Prince Edward Island, Quebec, contained *B. botulinus*, type A, while of 2 manured soils from Nova Scotia and Ontario, 1 gave type A and 1 type B culture.

The negative results obtained with soils collected from the vicinity of Montreal and the eastern section of Quebec may be due to the selection of samples. It is, however, possible that the lower portion of the St. Lawrence River basin, draining the Great Lakes, harbors relatively few spores of *B. botulinus* and in this respect is comparable to and continuous with the American Great Lakes region, which is known to be slightly infected.

Sixty-one virgin soil samples furnished 23, or 37.7%, while 30 specimens from manured or cultivated fields gave only 4, or 13.3%, toxic cultures. Nine vegetable specimens were found free from *B.*

botulinus; 14, or 60.8%, of the toxic virgin soil specimens contained *B. botulinus*, type A. These findings are in accord with the observations made in the United States, and it must, therefore, be concluded that *B. botulinus* occurs also in Canada in an ubiquitous distribution.

CONCLUSIONS

B. botulinus has not been demonstrable in the coast land soil of the Aleutian Archipelago.

B. botulinus, type A, and occasionally type B, has been cultivated in moraine, glacier and mountain soil collected around Lake Louise in the Canadian Rockies.

Soil samples obtained from the provinces of Prince Edward Island, Nova Scotia, Quebec, Ontario and British Columbia have also furnished positive cultures.

THE OCCURRENCE OF THE SPORES OF *B. BOTULINUS* IN BELGIUM, DENMARK, ENGLAND, THE NETHERLANDS AND SWITZERLAND. VI *

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Botulism has been recognized in Germany since 1735, but Bitter¹ wrote in 1921 that all attempts made in Europe to locate *B. botulinus* in nature had completely failed. In 1919 Semerau and Noack² expressed the opinion that the anaerobe occurred probably in regions or districts which from their geological or climatological location or incidental to contact with animal life and plant growth offered an environment suitable for multiplication and persistence. With the exception of the studies of van Ermengem³ and Bitter, no data were available to support the hypothesis of Semerau and Noack. In view of the American findings, it suggested itself to examine a number of soil specimens collected in European countries in which human botulism had occurred. Such an undertaking was also prompted by a desire to compare the *B. botulinus* strains of the Old World with those of the New World. It can be stated without any fear of contradiction that nothing definite is known with regard to the various strains of *B. botulinus* which have been isolated in Europe. The early descriptions of van Ermengem and Leuchs⁴ have with some slight modifications been copied by most of the recent writers. Moreover, the original strain of van Ermengem has been lost and fourteen so-called cultures of *B. botulinus* received from Germany during the last year have been found nontoxic, containing *B. sporogenes* or *B. centro sporogenes*. The only evidence which indicates that *B. botulinus*, type B or type A, is encountered in Germany is furnished by these observations: 1. The antitoxin prepared by the Institute of Infectious Diseases, Berlin, and supplied to us by Prof. Claus Schilling neutralizes the toxin of every American type B strain. 2. The Lister Institute of

Received for publication, July 26, 1922.

* This study was aided by grants from the National Canners Association, the Canners League of California and the California Olive Association.

¹ *Ergebn. d. allg. Path. u. path. Anat.*, 1921, 19, II, p. 762.

² *Ztschr. f. klin. Med.*, 1919, 88, p. 304.

³ *Ztschr. f. Hyg. u. Infektionskrankh.*, 1897, 26, p. 1.

⁴ *Ibid.*, 1910, 65, p. 55.

Preventive Medicine forwarded for comparison 2 cultures of *B. botulinus* originally received by them from the Institute of Infectious Diseases, Berlin. One culture (No. 95) was nontoxic, while the other (No. 94) furnished a pure growth of *B. botulinus*, type A, which is identical with the 70 American type A cultures. It has been impossible to determine whether this culture is a descendant of the original one isolated by Gaffky⁵ from the white beans responsible for the Darmstadt outbreak. In 1910 it had been demonstrated by Leuchs⁴ that the strain isolated by Gaffky differed immunologically from van Ermengem's original culture. On a comparative basis it appears not unlikely that Gaffky's culture was identical with what is known today as *B. botulinus*, type A, and if so is probably the only type A culture which has been isolated in Europe. It was expected that the examination of soil specimens from Europe might possibly confirm or enhance this interpretation.

Besides Germany, a number of European countries have reported outbreaks of botulism. For example, van Ermengem⁶ described 2 outbreaks which occurred in Belgium, 1 at Ellezelles in Hainau, and 1 at Jseghem in West Flanders. The observations reported by Madsen⁷ and Hoeg⁸ dealt with intoxications which followed the consumption of spoiled fish in Denmark. One outbreak each has been reported from the Netherlands and Switzerland. Unfortunately Blattmann,⁹ who described cases of botulism due to smoked ham in Switzerland, failed to mention the exact location of the outbreak. One botulism observation was made in England¹⁰ in 1860, and one in France¹¹ in 1875, in which it was stated that the poisonous sausages had been prepared in England. However, according to Savage¹² "botulism is unknown, or at least unrecognized," in England. This statement, made by the best known English authority on food poisoning and food infections, prompted the desire to examine soil specimens from England. On account of the supposed absence of botulism it was suspected that the soil of this country contained either none or, at most, very few spores of *B. botulinus*. Furthermore, by analogy with conditions in the United States it was reasoned that the heavily populated areas of the British Isles and

⁵ Lefar's Handbuch d. technisch. Mykologie, 1905-08, 2, p. 453.

⁶ Handb. d. pathog. Mikroorganis., 1912, 4, p. 918.

⁷ Centralbl. f. Bakteriöl., I, Ref., 1905, 37, p. 373.

⁸ Hospitalstid., 1915, 58, p. 300.

⁹ Cor.-Bl. f. Schweiz. Aerzte, 1909, 39, p. 18.

¹⁰ Brit. and For. Med., Chir. Rev., 1860, 25, p. 142.

¹¹ Ann. hyg. pub., 1875, 43, p. 472.

¹² Food Poisoning and Food Infections, Cambridge, 1920, p. 8.

of Europe would harbor few spores on account of the century-old manuring and tilling of the soil, and if present in such an environment that only type B would probably be encountered. That this assumption is correct is apparently proved by the findings reported in the next paragraph.

EXPERIMENTAL DATA

The technic employed for the study of the European soil specimens was identical with that reported in papers I, II, III, and IV of this series. This bacterio-geographical study would have been impossible without the hearty cooperation of several colleagues and friends. It is a great pleasure to acknowledge and to express again the sincerest thanks of the Hooper Foundation for the kind assistance rendered.

The observations made on 165 specimens are herewith briefly reported.

Belgium.—Through the assistance of Dr. Paul Fabry, exchange student at the University of California, 3 samples of soils were procured from Prof. E. Malvoz, at Liège, Belgium. Two cultures contained *B. botulinus*, type B.

Denmark.—Dr. H. O. Schmit-Jensen of Prof. C. O. Jensen's laboratory in Copenhagen, collected and forwarded in collaboration with the Lime Control Department of the Statens Planteavlslaboratorium, in June, 1921, 54 samples of soils, compost, etc., from Copenhagen and different parts of Denmark. The origin and character of the specimens are briefly as follows:

Eight garden soils [serum laboratory, botanical garden (2), Söborg, Gjentofte, Endrup, Trørød, Copenhagen].

Twelve field soils [serum laboratory, experiment field, celery field, night soil, cucumber field, cabbage field (2), Söborg, Gjentofte, Endrup, Trørød bean field and leek field; all near Ullerup, Amager].

Four sewage discharging berths.

Five composts, 1 roots and leaves, etc.

Twenty-five soils from Nordby; Sorvad; Risby, Snese; Haslund, pr. Randers; Sønderby; Borre, Moen; Saxild, Odder; Ll. Karleby, Roskilde; Hornskov, Sjørslev; Riisgaard, Grønbaek; Hauerbaek, Kjellerup; Ullerup, pr. Hurup; Baech, pr. Vonsbaech; Sommersted; Fodby, Fodby; Simonsgaard, Rutsker; Spagergaard, Nylars; Njøre, pr. Rødbj; Alsonderup, pr. Hillerød; Heldumboel, Lemvig; Aistrup, pr. Sulsted; Gravlev, Støvring; Birkely, pr. Ulsted; Arvelund, Nakskov; Hammerhus, Tørring; Aale.

The following cultures proved toxic on repeated examination:

Compost (3 yrs. old) Horticulture "Nykastrupgaard, Amager, *B. botulinus*, type B.

Soil from Aistrup, pr. Sulsted, *B. botulinus*, type B.

Soil from Birkely, pr. Ulsted, *B. botulinus*, type B.

A sample of remnants of roots and leaves in Kongelunden, Amager, near Copenhagen, soil from Hammerhus, Tørring, from Sorvad and from Hornskov, Sjørslev, produced weak toxins.

Summary: 8 garden soils, none; 37 field soils, 2 type B, 3 weak toxins; 4 sewage discharging fields, none; 5 compost, roots and leaves, etc., 1 type B, 1 weak toxin.

Total: 54 samples with 3 (6) or 5.5 (11.1) %, positive cultures.

England.—Dr. William G. Savage, County Medical Officer of Health, Somerset County Council, Weston-super-Mare, kindly organized the collection and shipment of 64 samples of soil from the following localities in England: Colchester, Rhondda, Durham City, Wokingham, Shincliffe, Shotley Bridge;

Penycae Ruabon, Eastbourne; Ealing; Gloucester; Brighton; Shrewsbury; Timperley (Cheshire); Derbyshire; Isle of Wright; Breinton (Hereford); Weston-super-Mare.

The following samples furnished toxic cultures:

Surface soil, near wood well enclosed by trees, at foot of the South Downs, near Eastbourne—*B. botulinus*, type B.

Soil from a heavy manured garden which had had road sweepings and horse manure dressings each year; 1 ft. deep in Ealing—*B. botulinus*, type B (surface soil sample of the same place, negative).

Surface soil on hillside, manured 1920, growing oats, South Downs, Brighton—*B. botulinus*, type B.

Soil 1 ft. deep in garden (kitchen), manured regularly with cow dung and horse manure, Shottle Hall, Derbyshire—*B. botulinus*, type B.

Surface soil from garden used for growing vegetables, heavily manured with farm manure each year and produces prolific crops, Breinton, near Hereford—*B. botulinus*, type B.

Surface soil from a field growing wheat at Shincliffe, and surface soil from sewage farm at Shotley Bridge, near Durham, produced weakly toxic cultures.

Summary: 47 garden and orchard soils (mostly manured), 4 type B, 2 weak toxins; 7 field soil, 1 type B, 1 weak toxin; 7 virgin soils, negative; 3 sewage farms, 1 weak toxin.

Total: 64 samples with 5 (9) or 7.8 (13.1) %, positive cultures.

The Netherlands.—Prof. D. A. de Jong, University of Leiden, Holland, furnished in March, 1922, 10 samples of soil which had been collected in Zeeland, Zuidland, Gelderland, Groningen, Utrecht, Drenthe, Friesland, Noord Holland, Noord Brabant and Limburg.

One specimen from Gelderland and one from Drenthe furnished *B. botulinus*, type B. The cultures prepared with the samples from Zeeland, Zuidland, Friesland and Noord Brabant were toxic. The filtrates produced typical botulism symptoms on feeding to guinea-pigs and mice, but neither type A nor type B nor a polyvalent antitoxin protected the animals against the intoxication.

Summary: 10 samples of soil, field or garden, 2 type B, or 20% positive cultures.

Switzerland.—Through the assistance of Prof. Dr. R. Burri, Schweizerische Milchwirtschaftliche und Bakteriologische Anstalt, Bern-Liebefeld, the laboratory received:

Sixteen samples of soil and dirt (meadows treated with liquid manure, 4; treated with cow's manure, 4; garden soil treated with cow's manure, 4; street dust, 2; and virgin soil from Alpine meadows of Canton Tessin and Bern, 2).

Eighteen specimens of vegetables (white cabbage; savoy, 2; white turnip, 2; dwarf peas; cauliflower, 2; white orach, preserved peas, yellow turnip, red beets, potato roots, silver beets, salad beets, Brussels sprouts; salad treated with liquid manure, 2).

The following specimens furnished toxic cultures:

Surface soil from meadow treated with liquid cow's manure, 1 *B. botulinus*, type B; 2 surface soil from cultivated field treated with cow's manure, 1 *B. botulinus*, type B, 1 weak toxin; 3 garden soil treated with cow's manure, 1 *B. botulinus*, type B; 1 salad from K  niz, treated with liquid manure, 1 *B. botulinus*, type B; 1 cauliflower, 1 *B. botulinus*, type B; 1 silver beets (Silber Mangold), 1 *B. botulinus*, type B; 1 red beets and 1 potato roots, 2 weak toxins; 1 Brussels sprouts, *B. tetani*.

Summary: 4 meadow soils treated with cow's manure, 1 type B; 4 cultivated fields treated with cow's manure, 1 type B, 1 weak toxin; 4 garden soil treated with cow's manure, 3 type B; 2 Alpine meadow soils, negative; 2 street dust, negative; 18 vegetable specimens from manured gardens, etc., 3 type B, 2 weak toxins.

Total: 34 samples with 8 (11) or 23.5 (35.2) %, positive cultures.

The essential results detailed in the preceding paragraphs are briefly summarized in table 26.

TABLE 1
RESULTS OF EXPERIMENTS WITH SOILS FROM VARIOUS EUROPEAN COUNTRIES

Country and Locality	Specimens	Total Number of Specimens Examined	Number of Toxic Cultures	Total Number of Typed Cultures	Type B	Un-typed	Percentage of Total Toxic Cultures	Percentage of Typed Cultures
Belgium: Unknown.....	Soils	3	2	2	2	—	—	—
Denmark: Copenhagen-Amager....	Garden soil (8); field soil (12); sewage berth (4); compost (5)	29	2	1	1	1	6.8	3.4
Different parts of the country	Field soils	25	4	2	2	2	16.0	8.0
England: Different parts.....	Garden and orchard soil (47); field soil (7); virgin soil (7) and sewage farms (3)	64	9	5	5	4	13.1	7.8
Netherlands: Different parts.....	Meadow soil	10	6	2	2	4	(60.0)	(20.0)
Switzerland: Vicinity of Bern and one Canton Tessin	Soils (16) and vegetables (18)	34	11	8	8	3	35.2	23.5
Total.....		165	34	20	20	14	20.6	12.7

A total of 165 soil and vegetable specimens have been examined. Thirty-four, or 20.6%, of the samples furnished toxic cultures, but only 20, or 12.7%, of them have been identified as toxins of *B. botulinus*, type B. It must be stated at once that the cultures prepared with the European specimens exhibited one striking peculiarity, namely, the toxins generated in peptic digest-liver broth and injected into guinea-pigs in 2 c c amounts failed to kill in less than 48 hours. In fact, most of the toxins were not fatal until 3 to 4 days had passed. Previous experience had taught that these weakly toxic cultures were the probable result of the presence of relatively few heat-resistant spores. This interpretation was further supported by the observation, repeatedly made, that the heating of these soil suspensions at the temperature of the Arnold sterilizer for more than 15 minutes invariably produced nontoxic enrichment cultures. In the experiments described in previous

papers this had been shown to be the case only when the original number of spores in the soil was a small one. Isolation and purification of *B. botulinus* from European soil samples were therefore found exceedingly difficult. Only one strain has thus far been enriched sufficiently to insure an isolation of a pure culture.

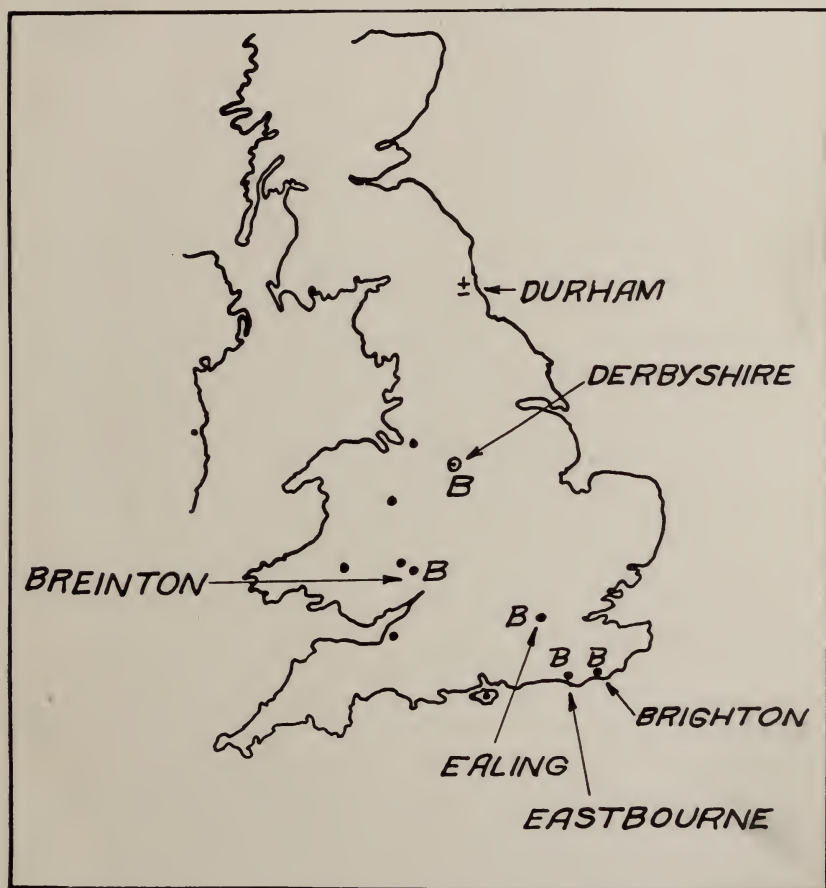


Chart 1.—Distribution of *B. botulinus* in England

The data secured with European field specimens confirm in a surprising manner the findings made in the United States, namely, that when type B is isolated the number of spores in the soil and its products is relatively small. Extensive cultivation suppresses *B. botulinus* and apparently eliminates type A. Every statement or conclusion which

has been made in the discussion dealing with the telluric distribution of *B. botulinus* in America can, therefore, in a general way, be applied to the conditions in Europe.

Only 3 of the 54 Danish samples contained *B. botulinus*, type B. This survey is sufficiently extensive to permit the conclusion that *B. botulinus* is rarely encountered, and then only in field soil or in composts. Garden or field soil heavily polluted with sewage gave nontoxic cultures.

From a geographical standpoint, *B. botulinus* is widely distributed in England, as indicated by chart 1. Considering, however, the fairly large collection of specimens studied, it must be concluded that the number of organisms is relatively small, because of 64 samples only 4 garden soils and 1 field soil contained *B. botulinus*, type B. One specimen from Breinton produced a potent toxin, and the organism was enriched in a fair state of purity. Cultures made from virgin soil samples were free from *B. botulinus* and allied anaerobes, as, for example, *B. sporogenes*, *Vibrio septique*, etc. Attention is again called to the absence of *B. botulinus* in the soil from sewage farms.

Six, or 60%, of the 10 soil specimens obtained from The Netherlands produced toxic cultures. Two cultures contained *B. botulinus*, type B, toxins. The purification of one strain is expected in the very near future. Four cultures killed guinea-pigs with characteristic symptoms on feeding (2 cc) in less than 20 hours, but neither type A nor type B, nor a polyvalent antitoxin protected the animals against this toxin. The repeated demonstration of a botulinus-like toxin in anaerobic enrichment cultures prepared with heated soil specimens suggests two questions:

1. Does the soil of The Netherlands harbor an anaerobe similar to that isolated by Bengtson¹³ in the United States from the larvae of *Lucilia Caesar*?

2. Are all clinical cases of botulism in Europe really caused by the poison of the same toxicogenic anaerobe which has been repeatedly isolated and studied in America?

These questions can be solved only when a specific antitoxin against the anaerobe of Bengtson is available. Insurmountable difficulties have been encountered in the numerous attempts to isolate this toxin-producing anaerobe from the enrichment cultures of the Dutch soil samples. It is, of course, known that the colonies of the anaerobe of Bengtson in mixed deep agar cultures resemble closely those of other

¹³ Public Health Rept., 1922, 37, p. 164.

nontoxic anaerobes or facultative aerobes. Several hundred colonies have been picked without meeting a toxic strain. These studies are being continued. Apropos of the question of whether or not all clinical cases of *B. botulism* in Europe are caused by the same anaerobe as that isolated in America, the following report of Bitter¹⁴ is of interest. He isolated in 1918 and 1919, from a pickled herring and a spoiled ham, 2 strains of an anaerobe, which he identified on toxicological, morphologic and biologic grounds as those of *B. botulinus*. An attempt to classify these strains by means of antitoxin furnished by the Institute of Infectious Diseases, Berlin, was not successful. In the opinion of Bitter this failure must be attributed in all probability to the deterioration of the serums. He did not note the least protective action against the toxins of his strains. In the light of the observations made in this laboratory this statement opens up a new and important aspect. One of the antitoxins used by Bitter is identical with that used by us, and it has been furthermore established that this antitoxin neutralizes the toxin of all the American type B strains. The failures of Bitter may, therefore, be the result of two conditions: (a) the serums used by him were really deficient in antitoxin, or (b) the cultures contained neither a type A nor a type B toxin but an immunologically new poison. Whether this toxin is that of the Bengtson's anaerobe or possibly a *B. botulinus*, type C, cannot be determined from the description of his bacteriologic analysis. It is stated that his strains are highly proteolytic. The anaerobe isolated from *Lucilia Caesar* lacks this property, and it is not unlikely that Bitter's cultures were impure. The observations made previously on four untypable toxic cultures of Dutch soil prompt these hypotheses. They deserve the earliest attention of investigators interested in the etiology of the clinical picture generally designated as botulism.

An analysis of the cultural results made on Swiss soil and vegetable specimens reveals a high percentage of positive cultures. Eleven, or 35.2% of the 34 samples produced toxic cultures, and 8, or 23.5%, contained *B. botulinus*, type B. Soil or vegetables derived from manured meadows, cultivated fields or gardens furnished the spore-containing samples. Two virgin soil specimens gave nontoxic cultures. This number is obviously inadequate to venture a comparison with the conditions observed in the United States. It is not unlikely that the soil of the Alps may harbor *B. botulinus* quite frequently. The factors which govern the distribution of *B. botulinus* in California are probably

¹⁴ Ref. 1, p. 791.

duplicated in Switzerland and may explain the high percentage of positive samples collected. Experiments are contemplated to verify these statements in the very near future.

The cultural analysis of soil and vegetable specimens obtained from Belgium, England, The Netherlands and Switzerland has definitely shown that *B. botulinus*, type B, exists in the Old World. This anaerobe has been found in countries in which human botulism is rare or unrecognized. As a rule, the spores are decidedly less numerous in a European than in an American soil specimen. Unfortunately, the process of purification of 2 strains has not advanced sufficiently to permit the testing of the heat resistance of the spores. However, a number of observations made incidental to the isolation of this anaerobe from toxic enrichment cultures have indicated that the spores fail to survive temperatures of 100 C. for more than from 10 to 15 minutes. Exposure of from 25 to 50 gm. of European soil containing the spores of *B. botulinus* to the temperature of boiling water of from 20 to 30 minutes produced nontoxic enrichment cultures. On the other hand, the resistance of these soil spores to temperatures below 100 C. is probably greater than is generally assumed from the studies of van Ermengem,⁶ who found that the spores of his strains withstood a temperature of 85 degrees for less than 30 minutes. Several soil specimens from Belgium, England, The Netherlands and Switzerland have been heated for 2 hours at 85 C., and repeated tests have furnished toxic enrichment cultures.

The heat resistance of the spores of *B. botulinus* has apparently little or no connection with the occurrence of human botulism in Europe. According to Bitter, only 2 outbreaks are known to have followed the consumption of canned foods—one due to home-canned string beans and one due to commercially packed burbot. About 68 of the known outbreaks in Belgium, Denmark, Germany and Switzerland have been caused by food which had not been subjected to severe processing temperatures. It is also pointed out that the prevalence of botulism in country districts is frequently due to inadequate preservation incidental to the careless and insanitary treatment to which the raw material is subjected by the rural population. Home slaughtering and preservation of pork products in the form of sausages is so widely practiced in Europe that it is not at all surprising to find that about one-half of the botulism outbreaks are caused by this type of food. Accidental contamination of these products with infected soil occurs only under the most insanitary conditions of rural districts. It must

therefore be expected that human botulism due to meat, fish or animal products occurs mainly in the regions in which the *B. botulinus* soil index is relatively high.¹⁵

CONCLUSIONS

B. botulinus, type B, has been demonstrated in soil and vegetable specimens collected in Belgium, Denmark, England, The Netherlands and Switzerland.

B. botulinus, type A, has been found consistently absent.

The spores are widely distributed, but they are neither numerous nor very resistant to heat.

¹⁵ Since this paper has been written, one of the authors (K. F. M.) has investigated an outbreak of botulism due to commercially packed potted meat at Loch Maree, Scotland. The wild duck paste was understerilized, exhibited no signs of spoilage and contained, according to the careful studies of Mr. Bruce White of Bristol University, *B. botulinus*, type A., and its toxin.

Dr. J. Williamson Tocher, Aberdeen, Scotland, has isolated *B. botulinus*, type A., from the spleen of a horse, which suffered from "grass sickness." Dr. J. B. Buxton of the Wellcome Research Laboratory has also demonstrated the toxin of *B. botulinus*, type A., in the blood serum of horses which have recovered from the same disease.

During the last 3 years, 2 outbreaks of botulism due to smoked ham have occurred in Switzerland. The toxin has been demonstrated but not typed in one instance.

THE OCCURRENCE OF THE SPORES OF *B. BOTULINUS* IN THE HAWAIIAN ISLANDS AND CHINA. VII *

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The predominance of *B. botulinus*, type A, in the Pacific Coast States is in striking contrast to that of type B in the Atlantic States, England and Europe. These differences in the distribution of types cannot be attributed to any topographic factors but coincide amazingly with the density of the population and agricultural activity. It naturally suggests itself to compare these observations with those found in specimens collected in other portions of the Western Hemisphere and in certain parts of the Eastern Hemisphere. The Hawaiian Islands have been chosen on account of their mountainous surface and their volcanic origin. The soil is very fertile, being formed by the disintegration of volcanic rocks and the decay of vegetable matter. These islands possess geologically a soil stratum which in all respects is very similar to that of the Pacific Coast and Sierra Nevada mountains. Furthermore, there is considerable agricultural activity in form of cane sugar and pineapple plantations.

The distribution of *B. botulinus* on the Asiatic continent has been studied on specimens obtained from China. This republic is heavily populated and is one of the most important agricultural countries of the Far East. Fertilization with sewage and animal manure has been practiced for centuries. The soil is known to contain *B. tetani* and other pathogenic anaerobes. A study of the soil specimens offers, therefore, data of comparative value.

EXPERIMENTAL DATA

Three series of specimens have been examined by the technic employed in similar studies. Guinea-pigs as well as mice have been used for the identification of toxic enrichment cultures.

Hawaiian Islands.—Through the courtesy of Mr. R. I. Bentley, President of the California Packing Corporation, 23 samples of soil and vegetables were procured from Wahiawa, of the Island of Oahu, in April, 1921. Cultures were made of the following specimens:

Received for publication, July 26, 1922.

* This study was aided by grants from the National Canners Association, the Canners League of California and the California Olive Association.

Five soil samples.

Eighteen specimens of banana roots, pineapple roots with manure (4); grass, papaia roots, tomato plants, parsley, radish, turnip, beet, strawberries, papaia fruit, banana, mulberries, cabbage, tops and roots of strawberries.

One sample of pineapple root, papaia root and mulberries produced weakly toxic cultures.

In January, 1922, one of us (P. S.) collected and made cultures of 16 additional samples of soil and feeds from the Island of Oahu. The cultures gave the following results:

One soil—Algarobagrove—not cultivated, about 150 ft. from a stable. *B. botulinus*, type B.

One surface soil, 6 inches from surface (equine botulism outbreak?), Farm I., *B. botulinus*, type B.

One deep soil, 1 ft. down near stable, farm 2, *B. botulinus*, type B.

One surface soil, same place, *B. botulinus*, type B.

One virgin surface soil—Tantalus, about 6 inches deep, *B. botulinus*, type A.

One virgin soil side road, 2 ft. down "Round Top," *B. botulinus*, types A and B.

One soil, same place surface soil, *B. botulinus*, type B.

One good grade rice straw, weak toxin. Manure pile, drainage, 200 ft. from stable, rice straw on farm 1, garden soil on "Round Top," lava deposits, soil from Tantalus, wet and dry tarrow patch in Manoa Valley (8), all negative.

Summary: 17 soil cultivated or manured, 5 type B; 2 virgin soil in mountain region, 1 type A and 1 type A and B; 20 vegetables and feed, 4 weak toxins.

Total: 39 samples with 7 (11) or 17.9 (28.2) %, positive cultures.

China.—In May, 1922, the laboratory received from Dr. Carl Ten Broeck, Peking Union Medical College, China, 52 specimens of soil collected from the following provinces and towns:

Thirty-two Chilili: Peking, 18 (near grave, 3; cultivated fields, 9; ground covered with trees, 2; temple ground near Lo Tz Lung An Tom, 1; cleared ground, 3). Near Temple Heaven, 14 (cleared ground, 6; ground covered with trees, 8).

Twenty Shansi: Near town of Kao Chia Chuang, 12 (cultivated field, 1; ground covered with trees, 8; freshly cleared field never cultivated, 1; cleared ground, 1; open forest, 1). Near town of Tzi Lao, 4 (hillside forest, 1; cleared ground covered with trees, 3). Near town of Chang Kou, 1 (cleared ground, 1). Near town of Ping Ting Chow, 2 (cultivated field, 2). Near town of Le Ping, 1 (cultivated garden, 1).

The cultures gave the following results:

Three cultivated field (no decayed vegetable matter present), Peking, 3 type B.

One temple ground near the door of Lo Tz Lung An Tom, Peking, 1 type B.

One near grave, 1 weak toxin.

Three ground covered with trees, near town of Temple Heaven, Peking, 2 type B, 1 weak toxin.

Two cleared ground, near town of Temple Heaven, Peking, 1 type B, 1 weak toxin.

Three ground covered with trees, near town of Kao Chia Chuang, Shansi, 1 type B, 2 weak toxins.

Two cleared ground, no decayed vegetable matter present, near town of Chang Kou, Shansi, 1 type A and 1 type B.

One hillside forest (open), near town of Tzi Lao, 1 type B.

One freshly cleared field, some roots, etc., Kao Chia Chuang, Shansi, 1 type B.

Two ground covered with trees, near town of Tzi Lao, 2 type B.

One cultivated field garden near town of Le Ping, 1 weak toxin.

Summary: 12 cultivated fields with and without decayed vegetable matter, 3 type B, 1 *B. tetani*; 21 grounds covered with trees, some without decayed vegetation, 5 type B, 3 weak toxins, 1 type A; 11 cleared ground with and without decayed vegetation, 2 type B, 1 weak toxin; 3 near grave, 1 weak toxin; 1 hillside forest, abundant vegetation, 1 type B; 1 open forest on steep hill (large amount of decayed pine needles) —; 1 cultivated garden, probably manure, 1 weak toxin; 1 temple ground, 1 type B (weak); 1 freshly cleared field, decayed vegetable matter present, 1 type B.

Total: 52 samples with 14 (20) or 26.9 (38.4) %, positive cultures.

Eleven, or 28.2%, of the 39 soil and vegetable samples collected in the Hawaiian Islands produced toxic enrichment cultures. The toxin of *B. botulinus* was identified in 7, or 17.9%, of the samples by anti-toxin neutralization. Five cultures contained *B. botulinus*, type B, while one was neutralized by a type A and another by a polyvalent antitoxin. Four cultures prepared from fruits, roots and rice straw yielded weak toxins. The spores *B. botulinus*, type B, were quite frequently encountered in the soil of the Island of Oahu. Surface as well as deep soil, and virgin as well as cultivated soil, carried the organism. *B. botulinus*, type A, was found alone only in one virgin soil specimen, but in another instance was present with type B in a surface mountain soil. Type B is the principal toxicogenic anaerobe. This observation apparently confirms our opinion that type B exists primarily in fertile soils which are favored by the decay of vegetable matter and abundant rainfall. The percolating rain water is probably responsible for the presence of *B. botulinus* spores in the deeper layers of the soil. Conditions favorable for generation of toxins in moldy straw, etc., are unquestionably present, and it is therefore not surprising to learn that one outbreak of equine botulism has been observed on the premises of the U. S. Aviation Field on Oahu. The laboratory studies presented in this paper have demonstrated the presence of the anaerobe in the rice straw and the soil of the farm on which the outbreak occurred. Major James S. Simmons, M. C., isolated the *B. botulinus* from the spleen of one of the horses. It is most unfortunate that at the time of the outbreak the suspected hay was not studied for the determination of the presence of *B. botulinus* toxin. Human cases of botulism have thus far not been recognized in the Hawaiian Islands.

The observations made on the 52 soil samples procured from two coast provinces in China duplicate those of the Hawaiian Islands,

except that the percentage of toxic cultures is somewhat higher, namely, 38.4%. One enrichment culture prepared from a specimen of soil derived from cleared grounds near Kao Chia Chuang was highly toxic and contained *B. botulinus*, type A. *B. botulinus*, type B, obtained in all the other specimens, is geographically widely distributed, but judging from the toxicity of the cultures, it is quite evident that the soil contains relatively few spores. The majority of toxins were fatal to guinea-pigs in not less than 36 to 72 hours. Grounds covered with trees, some showing decayed vegetation, yielded the largest percentage of toxic cultures. A few soils obtained from forests, etc., harbored the same type, and it is impossible to offer any final explanations for the predominance of type B in the samples examined. The evidence strongly indicates that this type is indigenous to the lowland of China. A study of soils collected in the Khingan Mountains or the interior of China, which is less heavily populated than the provinces of Chilili and Shansi, should be made in order to amplify the results thus far obtained. Geographic and telluric studies on the distribution of the various types of *B. botulinus* tend to emphasize one fact—the soils of the New World, and particularly the Cordilleran mountain system of North America, harbor type A, while countries with old civilizations, as, for example, China and Europe and some of the Atlantic and Middle Western States of America, contain *B. botulinus*, type B., either exclusively, or at least predominantly. The surveys thus far completed unquestionably offer scattered and fragmentary evidence of a distinct process of bacterial mutation, and it is along these lines that the distribution of *B. botulinus* should be further investigated. The problem of microbial evolution or the adaptive development of bacterial species to their environment receives in the light of the geographic studies on *B. botulinus* a new impetus, and it is hoped that additional work on the presence of this anaerobe in South America, India, Africa and Australia may be undertaken in the not too distant future.

CONCLUSIONS

B. botulinus, type B, is frequently found in the soils obtained from the Island of Oahu, in the Territory of Hawaii, and from the provinces of Chilili and Shansi, in China. Type A has been found only in two Hawaiian and in one Chinese soil specimen.

THE OCCURRENCE OF B. TETANI IN SOIL AND ON VEGETABLES. VIII *

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A number of enrichment cultures prepared in the course of the studies on the distribution of *B. botulinus* in the soil of the United States, Switzerland and China, produced on subcutaneous inoculation in guinea-pigs typical symptoms of tetanus. In every instance neutralization of the toxin was accomplished by the simultaneous injection of 1,000 units of commercial antitoxin. Unfiltered but centrifugalized cultures which caused tetanus were tested for *B. botulinus* toxin by feeding portions to guinea-pigs and mice. One sample of the soil from Maryland was found to contain the toxin of *B. botulinus*, type B, and that of *B. tetani*. The data, dealing with the distribution of the tetanus bacillus in 2,379 soil and vegetable samples collected in the United States, Europe and China, are the result of casual observations. No attempt has been made to study the factors necessary to demonstrate this bacillus in mass enrichment cultures. It is known that the toxicogenic capacity of *B. tetani* is greatly reduced in the presence of other aerobes and anaerobes, and it is quite possible that the conditions chosen for the study of *B. botulinus* were not always suitable for the production of a potent tetanus toxin. In order to stimulate a study of the distribution of *B. tetani* in nature, however, the few observations are briefly summarized in table 1.

TABLE 1
DISTRIBUTION OF B. TETANI IN VARIOUS STATES

Arkansas.....	2	uncultivated virgin soil samples
California.....		Horse trail on Mt. Lowe, near Los Angeles
Indiana.....	1	pasture and 1 vegetable (swiss chard)
Maryland.....	1	uncultivated virgin soil sample, together with <i>B. botulinus</i> , type B
Michigan.....	2	garden soil specimens
Minnesota.....	2	garden soil specimens
New York.....	3	virgin soils collected around Mt. Baker, near Saranac Lake
Rhode Island.....	1	cultivated field (corn) and on 1 spinach specimen
West Virginia.....	1	virgin soil specimen from the Alleghany Mountains
Wisconsin.....	3	soil specimens from a heavily manured celery farm near Milwaukee
Switzerland.....	1	vegetable (brussels sprouts) from the vicinity of Bern
China.....	1	cultivated field near Pekin

Received for publication, July 26, 1922.

* This study was aided by grants from the National Cannery Association, the Cannery League of California and the California Olive Association.

It will be noted from the data presented in this table that nine of the states of America which furnished enrichment cultures with *B. tetani* are topographically east of the Mississippi. No western soil material, with the exception of one (trail dust on Mt. Lowe, Calif.), produced cultures containing *B. tetani*. These differences are, on account of the large number of cultures involved, sufficiently striking to justify further discussion. Three hundred and ninety-seven specimens from 9 (Arkansas, Indiana, Maryland, Michigan, Minnesota, New Jersey, Rhode Island, West Virginia and Wisconsin) states mentioned in table 1 furnished 18 cultures, while 991 cultures prepared with similar samples from California, Oregon, Washington, Montana, Idaho, Nevada, and Wyoming gave only 1 culture containing *B. tetani*. During a study of 624 specimens which originated in California, special attention was paid to the occurrence of tetanus bacilli in mountain soil, but the negative results prompted the conclusion that the spores of *B. tetani* are rare in comparison to those of *B. botulinus*. As it is known that well-manured garden soil has yielded tetanus bacilli in the hands of various observers, it is rather surprising to find the numerous specimens of garden and manured field soil from Oregon, Washington and California free from this anaerobe. It has been suggested that these results are due to the technic employed, and in particular the method of testing the toxicity of the enrichment culture has been suspected as inadequate. In this connection it should be stated at once that the tetanus toxin in the enrichment cultures was always recognized in the course of the neutralization tests with *B. botulinus* antitoxin. As a rule, all 3 guinea-pigs developed tetanus. The criticism that the presence of tetanus spores cannot always be demonstrated in mixed cultures is probably true for a small number of specimens, but this must apply equally to the samples collected in the East and Middle West, as the same method was employed throughout the entire series of studies. The evidence permits, therefore, only one conclusion: *B. tetani* is less prevalent in the soil of western states than in that of the eastern and middle western states. It is naturally possible that permanent infestation of the regions originally free from it may occur through commercial and industrial agencies, just as certain sections of California (Sacramento delta) have become permanently infected with anthrax spores from the offal and residues washed down into streams from tanneries, hair factories and improper burial places of animal cadavers.

The demonstration of tetanus spores in garden soil and on vegetables collected in eastern territories, Switzerland and China is quite in accord

with the well-known facts established many years ago by Nicolaier,¹ Bossano,² Bisserié,³ Sanchez-Toledo and Veillon,⁴ Rabinowitch,⁵ and others. The association of tetanus spores with fertilization and manure has always been supported by an old theory derived from the French, which claims that the tetanus bacillus is a regular inhabitant of the intestines of the horse and other species of domestic animals. Even human feces have, according to Pizzini,⁶ in Italy, and Ten Broeck,⁷ in China, been found to harbor this anaerobe. According to the data presented in table 1, spores of *B. tetani* are found in virgin and uncultivated forest soil where plant material is undergoing fermentation and decay. It is, therefore, quite conceivable that this anaerobe can, just as *B. botulinus*, multiply in symbiotic relation with other anaerobes or aerobes, wherever protein material undergoes putrefaction. The presence of the tetanus bacillus in so many places outside the intestinal canals of animals suggests much more extensive breeding places than the animal body. Irrespective of the recent findings of Ninni,⁸ who failed to find the spores of *B. tetani* in the soil from mountainous regions and elsewhere free from the dejecta of domestic animals, in studying *B. tetani* careful consideration should be given the facts established for *B. botulinus*. The entire problem of breeding places of all pathogenic anaerobes deserves renewed investigation in the light of the data presented in the papers dealing with the distribution of *B. botulinus* in nature.

CONCLUSIONS

The spores of *B. tetani* are frequently encountered in well manured, cultivated or garden soil, and on vegetables obtained from several states east of the Mississippi, Switzerland and China. In the United States even virgin forest soil has yielded cultures of *B. tetani*. The soil of the western states is relatively free from this anaerobe.

¹ Deutsch. med. Wchnschr., 1884, 10, p. 842; Beitr. z. Aetiologie des Wundstarr Krampfes, Göttingen, 1885.

² Rev. de méd., 1889, 9, p. 102.

³ Thèse, Paris, 1894, No. 126.

⁴ Semaine méd., 1890, 10, p. 45.

⁵ Arch. f. Hygiene, 1907, 61, p. 103.

⁶ Riv. d'igiene e san. pubbl., 1898, 10, p. 170.

⁷ Personal communication, and Jour. Exper. Med., 1922, 36, p. 267.

⁸ Ann. d'igiene, Rome, 1920, 30, p. 736.

A SAFE METHOD FOR SECURING ANAEROBIOSIS WITH HYDROGEN. IX *

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In the course of some quantitative studies on the growth of anaerobes in various plant and animal products, one of the authors (C. C. D.) has, during the past year, employed 13 large anaerobic jars. They have been operated in accordance with the principles proposed by Laidlaw¹ and constructed according to the descriptions published by McIntosh and Fildes.² It is the purpose of this paper to enumerate briefly the various details and operations which have eliminated the danger of explosions. The arrangement of the equipment as used in this laboratory is shown in fig. 1.

It will be seen from the illustration that the jar is not unlike that described by Brown.³ A few alterations in construction are indicated in the photograph and are briefly summarized as follows: (1) The wires conveying current to the heating coil are soldered⁴ to short pieces of "Pyrex" glass tubing, which are then cemented into a rubber stopper (A). This method of introducing the lead wires readily permits the use of multiple stranded copper wire (as ordinary, flexible electric light cord), minimizing the chance of breakage. The wires should be looped over and tied to the side of the glass tubes on the outside of the jar top to prevent any strain from occurring at the soldered joint. (2) A shield of asbestos sheet (B) is placed over the upper surface of the coil to prevent the heat from cracking the jar top. (3) Instead of mica as used by McIntosh and Fildes, asbestos washers are placed at these points (C) to deflect the heat from the inner ends of the rubber stoppers.

In several papers,⁵ it is emphasized that the anaerobic apparatus embodying the principle of Brown's jar produces excellent results, but

Received for publication, Aug. 7, 1922.

* This study was aided by a grant from the National Canner's Association, the Canner's League of California and California Olive Association.

¹ Brit. Med. Jour., 1915, 1, p. 497.

² Lancet, 1916, 1, p. 768.

³ Jour. Exper. Med., 1921, 33, p. 677.

⁴ Modification of method described by McKelvy (Jour. Am. Chem. Soc., July, 1920). Surface of glass to be soldered coated with metallic platinum by dipping repeatedly in alcoholic PtCl₄ solution and heating to a dull red heat.

⁵ Smillie: Jour. Exper. Med., 1917, 26, p. 59. Berg: Jour. Am. Vet. Assn., 1922, 14, p. 189. Gochenour: Ibid., 1922, 14, p. 301.

it is also indicated that in all the adaptations or modifications some danger of explosion still persists. The risk of igniting the hydrogen in the jar from the catalyzer itself seems to be eliminated by the use of the wire gauze employed in the construction of the capsule and electrically heated catalyzer described by McIntosh and Fildes. In a recent note, Brown ⁶ has, however, pointed out that an explosive mixture still



Figure 1

exists in the jar, which may be ignited by a temporary shorting or breaking of the wires, while the circuit is closed. By operating the apparatus in accordance with the method about to be described, the writers believe that the danger of a hydrogen explosion occurring in the jar may be entirely eliminated.

⁶ Jour. Exper. Med., 1922, 35, p. 467.

TECHNIC EMPLOYED IN OPERATING JAR

The flange of the jar top (D) is coated to a depth of about $\frac{1}{4}$ inch with plasticine modeling clay, which is spread to form a smooth layer of even depth. The top is pressed firmly in the clay and the jar is then evacuated through the stop-cock (E). As a rule, the plasticine joint around the top leaks considerably when the jar is first attached to the vacuum pump, but as soon as the pressure within the jar is reduced, the top will set more firmly, forcing out a ridge of plasticine. By spreading the projecting clay smoothly over the interstice between the jar and top, a tight seal may readily be obtained. Plasticine exposed to 37 C. becomes brittle, and frequent renewal is advocated. When evacuation of the jar is completed, the stop-cock (E) is closed and the jar connected to the hydrogen supply. Hydrogen is passed in under very slight pressure until the pressure within the jar reaches that of the hydrogen supply. A convenient apparatus for controlling the gas flow is shown (F, G and H) and will be described in detail in a subsequent paragraph. An electric current of suitable strength is passed through the heating coil until a satisfactory anaerobiosis is secured.⁷ The jar is permitted to remain connected with the hydrogen supply during this operation, after which the stop-cock is closed and the jar disconnected for incubation.

A Kipp generator will supply hydrogen at sufficient pressure to give satisfactory results with this method. Where hydrogen can be obtained in cylinders, a much more convenient and dependable supply is afforded. A reducing valve is desirable but not absolutely necessary with the gas controlling apparatus shown in fig. 1, which consists of the following parts: (F) flask (open at top) containing a loop of pressure tube submerged in water; a slit about $\frac{3}{4}$ inch long in the rubber tube in the flask (F) functions as a safety valve; (G) inverted flask fitted with a three-holed rubber stopper, accommodating a hydrogen intake tube (short), a hydrogen discharge tube (long), and a drain tube (short) with stop-cock. H indicates the hydrogen supply. This apparatus serves as a trap to remove any water which may be carried over from the flask (F).

In practice, the "safety-valve" device also serves as a visible index of the gas flow. A rapid flow of gas is permitted until the vacuum in the jar is overcome, permitting the filling of the jar in a few seconds. When the jar has been filled, as indicated by the emission of gas at the slit, the flow of hydrogen may be reduced (until only a tiny stream of bubbles escapes) during the remainder of the operation.

If the jar is to be incubated much above room temperature, it is advisable to diminish the pressure in the jar, unless a clamp is applied to hold down the tops. The removal of a few cubic centimeters of gas with a pump suffices to prevent the top from being displaced by expansion. It is recommended that the use of a clamp to hold the top in position be avoided until it is disconnected from the electric current and the hydrogen supply. The danger incidental to overfilling or ignition is reduced to a minimum when the top is only retained by the viscosity of the plasticine gasket. This fact is strongly emphasized by an experiment in which an explosion has purposely been induced.

⁷ No experiments have been made to determine what temperature is necessary to activate the catalyzer, nor exactly how much time is needed to deoxygenize completely the jars at the temperature employed. Preliminary tests with illuminating gas have suggested the following heating capacity, which has been used with entire satisfaction: The heating coil consists of 3 feet of nichrome wire (No. 30 B. and S.). It is operated by connecting in series parallel with two 50 watt lamps on a 110 volt a. c. circuit. The current is usually left on about 20 minutes, one of the lamps being cut out 2 or 3 times after starting, to avoid unnecessary heating of the jar and contents.

It is evident that the operation of the jar described produces at the time the electric current is passed through the coil, a mixture of gases which is so deficient in oxygen as to be practically nonexplosive. This fact has been conclusively demonstrated by the following experiment:

An anaerobic jar similar to that shown in fig. 1 (except that the catalyzing unit was removed) was sealed, exhausted, and filled with hydrogen as described. An attempt was then made to ignite the gas within the jar by passing an electric current (110 volts, a. c.) through a fine steel filament within the jar. The filament fused, breaking the circuit and consequently inducing a spark of considerable intensity, but no explosion resulted. The test was then repeated, using a jar which was poorly exhausted, the internal pressure being about 30 cm. before the hydrogen was introduced. An explosion of considerable violence took place. It must be stated at once, however, that although the top of the jar was thrown upward with great force, the jar itself remained intact, and no shattering of glass occurred. Obviously, if the top had been clamped on the jar, the destructive force of the explosion would have been greatly increased.

Since the absolute safety of this method depends on the removal of a large proportion of the oxygen by evacuation, it appeared advisable to determine whether or not its application would be of value in laboratories equipped with relatively poor vacuum pumps. With this object in view, a jar was evacuated to 30 cm. pressure (which is readily obtainable with an ordinary aspirator, or water pump), and then the partial vacuum was replaced by hydrogen. This operation was repeated, after which the jar was submitted to the ignition test described. No explosion occurred.

It can be stated without any fear of contradiction that the anaerobe jar constructed according to the principles of Fildes and McIntosh^{*} is the most dependable equipment, which produces regularly a satisfactory degree of anaerobiosis. Problems dealing with the quantitative estimation of bacterial growth of *B. botulinus*, *B. tetani*, and so forth, in mediums of varying composition and hydrogen-ion concentration have been made accessible by the use of these jars. Excellent growth has been obtained on the surface as well as in the depth of plain liver or milk agar plates. Papers which are in process of preparation indicate that the study of the physiology of sporulation, enzyme production and colony characteristics has been materially enhanced by the apparatus and operations described. In fact, the isolation and purification of delicate anaerobes have been greatly facilitated by the use of sheep blood agar plates in a reliable anaerobic jar.

From the foregoing, it is concluded:

1. That by means of the technic here described, dependable anaerobiosis may easily be secured in jars of the type described, with no

^{*} Brit. Jour. Exper. Med., 1921, 2, p. 153.

danger from possible ignition by the electric current employed in activating the catalyzer ; and that in the absence of an efficient vacuum pump the safety of the method may be insured by "washing" the jar several times with hydrogen after a partial evacuation.

2. That by dispensing with the use of a clamp to hold the jar top in position, the danger arising from an explosion from any possible cause may be reduced to a minimum.

3. That jars constructed and operated as described are capable of producing anaerobiosis, with a negligible risk to the operator.

SOME OBSERVATIONS ON THE PATHOGENICITY OF *B. BOTULINUS*. X

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During the last four or five years, owing to the apparently increasing number of cases of food poisoning among human beings and animals definitely recognized as being due to the contamination of foods with *B. botulinus*, an intensive study has been undertaken of various phases of this question. More particularly is this true in the United States where, because of the increased use of home canned foods urged on the population during the World War by the government, there seem to have been more of these outbreaks. A study of the rapidly growing literature on this subject testifies to the fact that much important work has been accomplished in elucidating many of the problems, both scientific and practical, which confront those responsible for and interested in safeguarding the public health. It appears to the writers that one of the most important questions in this connection which has received insufficient attention concerns the pathogenicity of the organism itself when introduced into the animal body.

The study of this particular problem may properly be divided into three parts: (1) The injection or ingestion of detoxified *B. botulinus* spores or bacilli; (2) the injection or ingestion of the spores or bacilli together with a minute dose of *B. botulinus* toxin (or other substances) insufficient by themselves to produce symptoms of botulism; (3) the latency of injected or ingested spores of *B. botulinus* in the animal body where such spores, due to various causes, may later germinate, multiply or be freed of their toxin and produce symptoms of the disease.

The first of these conditions will concern us principally in this paper, though perhaps the third part is of equal importance, and being intimately connected with the first, it will be necessary to touch on its various phases from time to time. However, a more complete discussion of the last two problems will be reserved for a later paper.

Frequently spore-containing foods are eaten which may have been sufficiently heated to destroy the toxin but not the spores. As such foods may contain few or many viable spores, it becomes of the

gravest importance to increase our knowledge as to the possible pathogenicity of these detoxified spores thus consumed. In considering this possibility, we are immediately confronted by the following questions: Does the introduction of detoxified spores into the animal body produce symptoms or death from botulism? If so, how many such spores are necessary to produce these effects, and finally, what is the mechanism of the pathogenic manifestation of these organisms?

The work dealing with the effects of the injection of unheated *B. botulinus* cultures does not concern us for the moment, but a review of the few investigations that have been made with detoxified spores shows us that to produce toxic symptoms a larger number of spores is required when fed than when injected by other routes.

The early experiments of van Ermengem¹ would tend to demonstrate that "*B. botulinus* is absolutely incapable of reproducing itself in the animal body." His cultures of the various organs and contents of the intestinal tract were negative. He admits, however, the possibility of growth in the intestinal tract under proper, but unknown, conditions. Other European workers, following his investigations, came to similar conclusions. These workers did not use a medium in the production of their spores capable of giving a maximum toxicity. Later Dickson² stated that the toxin is never formed within the body, and he bases his conclusion on his belief that the toxin could not be formed at a temperature in excess of 28 C. Graham and Brückner³ state that in cases of forage poisoning "a fatal intoxication was suggested by repeated failures to recover the organism from the blood in the course of fatal and experimental disease, while the brain, cord, liver, blood, spleen, mesentery, and suprarenals from 26 experimental cases of fed and subcutaneously injected animals were all negative."

Thom, Edmondson and Giltner⁴ originally failed to produce symptoms of poisoning by feeding or injecting detoxified spores of *B. botulinus*, but later⁵ these authors were successful. They caused death in 1 guinea-pig in 13 days, by the subcutaneous injection of only 30,000,000 spores of the Boise strain, though 31,000,000 spores of the Nevin strain produced no effect. In their feeding experiments, they produced death in 1 animal with 78,000,000 spores (Nevin strain) in 7 days, though subsequently from 75,000,000 to 112,500,000 spores of the same strain failed to produce any result. The Boise strain was fatal in from 5 to 7 days in doses of 180,000,000 to 192,000,000 spores. These authors also conducted numerous experiments based on the work of Bullock and Cramer⁶ who injected calcium chloride at the same time as the spores, or at varying intervals thereafter, thereby reducing the time survival of their guinea-pigs. They admit that their results are irregular, but are convinced of the pathogenicity of the detoxified spores of *B. botulinus*. They also admit the possibility of growth in the animal body, as well as the absorption of intracellular toxin from the spores.

¹ Ztschr. f. Hyg. u. Infektionskr., 1897, 1, p. 26.

² Canad. Med. Assn. Jour., 1918, 8, p. 903.

³ Jour. Bacteriol., 1919, 4, p. 1.

⁴ Jour. Am. Med. Assn., 1919, 73, p. 907.

⁵ Arch. Int. Med., 1920, 26, p. 356.

⁶ Proc. Roy. Soc., London, Ser. B, 1919, 90, p. 513.

During their investigation of an outbreak of olive poisoning, Armstrong, Story and Scott⁷ inoculated a guinea-pig subcutaneously with a heated suspension containing "some 300,000,000 bacilli . . . and numerous spores," with no result. Heated cultures were fed with negative results, while washed spores were fatal when force fed, but not when fed on grass or meal. These authors by experiment estimated 1 olive to contain "a minimum of 1,300,000,000 bacilli, presumably spore bearers" and believe from their work with animals that "a bite of olive containing this number of viable organisms, if capable of multiplying and forming toxin in the alimentary tract, should have caused serious infection."

Burke, Elder and Pischel,⁸ from a study of numerous outbreaks of botulism in man, conclude that the ingestion of toxin free spores, particularly in small numbers, does not cause this disease. Orr⁹ shows that the feeding or injection of massive doses of toxin free spores produced botulism in guinea-pigs and mice. He caused the death of 1 guinea-pig in 5 days and only "weakness" in another, by feeding 90,000,000 spores. One animal died in 33½ hours after the ingestion of 200,000,000 spores. Of 2 animals injected subcutaneously with 45,000,000 and 50,000,000 spores, the first died in 249 hours and the other in 50 hours. He believes that the spores grow in the body and produce toxin which can be demonstrated by a precipitin test, as well as by direct toxicity tests of the emulsified organs. The counted spores in some of these experiments were fed on bread, which would appear to be a source of error in the estimation of the number of spores actually entering the digestive tract. The irregularity of Orr's results is similar to that obtained by Thom, Edmondson and Giltner. Geiger, Dickson and Meyer,¹⁰ in a complete analysis of the question of botulism, more particularly in that portion of their work devoted to its epidemiology, discuss the possible pathogenicity of the detoxified spores. They imply some doubt as to the complete detoxification of the spores used by some of the other workers who produced botulism in animals. They, themselves, obtained negative results when heavy spore emulsions were mixed with the specific antitoxin, washed, and then heated at 80 C., and they suggest that further evidence should be obtained in this matter by careful scrutiny of further outbreaks before assuming that detoxified spores naturally ingested by human beings are pathogenic. Furthermore, they cite several human cases to show that those who ate the raw spoiled food died, while those who ate the same food heated showed no symptoms. However, they add that the experience gained by a study of these outbreaks neither supports nor refutes the results of these experimental investigations as the spore content of these heated foods was unknown.

Graham, Schwarze and Boughton¹¹ have recently shown that freshly drawn milk from artificially infected udders of cows is, under certain conditions, lethal for guinea-pigs, but the elaboration of toxin in the animal udder tissue or mammary secretion could not be established. These authors conclude from these and numerous feeding experiments that the possibility of *B. botulinus* toxin being excreted in the milk of lactating animals consuming contaminating feed is remote, though the contamination of milk by the feces or other sources furnishing spores of *B. botulinus* is not to be disregarded.

⁷ Pub. Health Repts., 1919, 34, p. 2895.

⁸ Arch. Int. Med., 1921, 27, p. 265.

⁹ Jour. Infect. Dis., 1922, 30, p. 118.

¹⁰ Bull. U. S. Public Health Service, in press.

¹¹ Am. Jour. Pub. Health, 1922, 12, p. 659.

Several of the following factors may play an important part in estimating the value of and in comparing the results obtained by different workers along these lines. It is important to know the method of determining the number of viable spores, the approximate number of vegetating forms present, the mediums from which they were obtained, their age, the methods of freeing them from their toxin; also the time elapsed between the detoxification and counting, as well as the injection, the method employed in feeding, and finally the purity of the spores after these various manipulations. Few workers mention all of these conditions or give controls for toxicity of the culture from which the spores were obtained, or for complete detoxification at the moment of injection.

As the pathogenicity of bacteria for the animal body as a rule depends on their ability to invade and reproduce themselves in the tissues, it is unfortunate that so few workers have studied culturally the organs of experimental and clinical cases of botulism. Orr¹² recovered *B. botulinus* from the spleen and liver. He also found the liver and brain of his experimental animals toxic for mice, and later records a few positive findings of *B. botulinus* in the liver and spleen together with entirely negative results in some of his fatally infected animals injected subcutaneously and fed large doses. Dickson¹³ recovered the organism from the spleens of a few of his animals injected intravenously and van Ermengem, among the first, remarked that *B. botulinus* could be recovered only from the organs of animals in which a large quantity of spore material had been injected by this route. The same worker,¹⁴ as well as Ornstein¹⁵ and Graham,¹⁶ also reports having found *B. botulinus* in the spleens of fatal cases of botulism.

B. botulinus has been found by several investigators in the dejecta of animals experimentally fed with material containing toxin and spores of this organism, but we have seen no record of the finding of the toxin in the stools of clinical or experimental cases. Meyer and Geiger¹⁷ give in detail their studies of the stools and tissues of various fatal cases of botulism, in man and in cattle. In the former, they obtained both types of the organism from the stools at varying periods after the causative meal was eaten, as well as from the wall of the jejunum in one case and from several parts of the intestine in another, but their attempts to recover the organism from the spleens in three cases were negative. The foregoing positive findings were reported, as well as "isolations from the liver, mesenteric lymph nodes, etc.," of cattle. Records and Vawter,¹⁸ in a study of diseases of cattle report the recovery of both types of *B. botulinus* from infarcts of the liver.

Recently Tanner and Dack¹⁹ published positive findings of *B. botulinus* in 2 out of 10 samples of stools from normal persons. Dubovsky and Meyer,²⁰ in a search for this organism from the stools of 45 normal persons, reported entirely negative results. In view of these results and in considering the reported positive findings from laboratories where constant work of this character is in progress, the possibility of contamination even by experienced workers should always be borne in mind.

¹² Abstr. Bacteriol., 1920, 4, p. 10.

¹³ Jour. Am. Med. Assn., 1918, 71, p. 518.

¹⁴ Ztschr. f. Hyg. u. Infektionskr., 1897, 26, p. 4.

¹⁵ Ztschr. f. Chemotherap., O., 1913, 1, p. 458.

¹⁶ McCaskey: Am. Jour. Med. Sc., 1919, 158, p. 57.

¹⁷ Pub. Health Rept., 1921, 36, p. 1313.

¹⁸ Jour. Am. Med. Assn., 1922, 79, p. 132.

¹⁹ Jour. Am. Vet. Med. Assn., 1921, 60, p. 155.

²⁰ Jour. Infect. Dis., 1922, 31, p. 501.

Dubovsky and Meyer,²⁰ in a series of human cases seem to have taken cultures from more of the organs than we have found reported elsewhere—they studied the spleen, liver, brain, lungs, kidneys and wall of the jejunum, ileum and colon, besides the intestinal contents. In 4 cases studied they found *B. botulinus*, types A and B, in the intestinal contents, and in various parts of the wall itself in cases from the outbreak at Florence, Arizona; and once in the liver and intestines of one case in the Healdsburg outbreak, though the findings for the other organs were negative. Their direct toxin tests from all this material also gave negative results. The same authors also obtained cultures from the organs and intestinal contents of horses, cattle and chickens, as well as from the crops of the latter. While complete findings are not given, they mention having recovered the organism from the spleens and livers of chickens, also frequently from their crops and intestines. The livers and mesenteric lymph nodes from 2 cows were positive. They admit in their article, which embraced work undertaken over a year ago, that it had not been proved that there is an etiologic relationship of these organisms to the lesions or organs from which they have been isolated and comment on the significance of the findings from the crops and intestinal contents of chickens which swallow large quantities of soil. In the light of these findings, Dickson's failure to recover *B. botulinus* from the colon and ileum of 250 grain fed hogs does not seem remarkable, providing these hogs were not fed other foods which might have been grossly contaminated with spores of *B. botulinus*. It is possible, however, that these negative results are due to the taking of cultures from insufficient fecal material, as the work of this laboratory along these lines has shown that occasionally these spores may be found in the feces of normal guinea-pigs and rabbits as well as in grain.

The records of the study of the pathology of the various organs in human beings and animals which have shown symptoms of botulism poisoning are even more meager than the reports of cultural studies. A few European workers and Wilbur and Ophüls,²¹ Armstrong, Story and Scott,⁶ Meyer and Geiger,¹⁷ and Dickson²² in this country, mention the histologic changes found. Most of these authors noted a characteristic type of thrombus formation in the vessels of the brain. Dickson²² believes that these thrombotic changes are not responsible for the symptoms of botulism, as they are rarely found in animals which have died within 48 hours after receiving an injection. In a recent important communication, Warthin²⁴ describes not only the lesions encountered in the brains of 6 human cases studied, but remarks that "all presented similar features of antemortem gas production in the brain and formation of small gas cysts containing bacilli morphologically resembling *B. botulinus*. These cases present evidence that human botulism is an infection as well as an intoxication."

Some of the negative results in the search for *B. botulinus* in the organs by the various workers may be due to the cultural methods employed, to the delay in taking cultures or previous chemical treatment of necropsy material from human cases. However, an analysis of the results obtained as well as a consideration of the opinions expressed

²¹ Arch. Int. Med., 1914, 14, p. 589.

²² Monograph, Rockefeller Institute, No. 8, 1918.

²³ Jour. Am. Med. Assn., 1921, 77, p. 483.

²⁴ Ibid., 1922, 79, p. 71.

shows an increasing probability that further study along these lines will definitely decide the question as to the growth of *B. botulinus* in the animal body.

TECHNIC

The spores of *B. botulinus* used were obtained from cultures in veal peptic digest gelatine, from 2 to 4 months old. They were washed from 1 to 3 times in 60 to 75 cc of saline phosphate, suspended in the same medium, heated 1 hour at 80 C. in a sealed tube at the same time as the supernatant culture fluid and final wash water, and injected or fed immediately thereafter. Guinea-pigs were used in our experiments unless otherwise stated. They were usually fed while fasting and invariably with 1 cc of suspension by means of a pipet attached to a rubber tube held in the operator's mouth. As a control for toxicity of the culture from which the spores were obtained and for the detoxification of the spores themselves, both heated and unheated supernatant culture fluid and heated and unheated final wash water were injected subcutaneously into guinea-pigs. This precaution was always taken and, in order to save repetition, the protocols of these injections for each experiment will not be given. Exceptions to this technic are to be noted in the experimental data given in tables 1 and 2.

Direct microscopic spore counts checked by cultivated dilutions in both beef heart and agar were made immediately after heating and the approximate number of vegetative forms noted. The purity of the spores was verified later from the cultivated dilutions.

A rather full consideration of the technic employed in the necropsies and taking of culture from the organs of our animals may not be out of place in view of the negative results obtained by the early workers and the paucity of records of positive findings of the organism in the various organs by recent investigators.

Guinea-Pigs.—It is essential to use animals from a healthy stock in order to avoid unobserved symptoms or death from an intercurrent infection, with consequent distribution of various contaminating organisms throughout the body. As it is possible, where *B. botulinus* is a common soil anaerobe or when the guinea-pigs have been kept in cages possibly grossly contaminated with *B. botulinus*, to find this organism occasionally in the feces and rarely in the organs of apparently normal animals, it is imperative that each guinea-pig used should be from strictly fresh stock and placed and maintained separately in small cages which have been adequately sterilized. This we have done.

Necropsy.—Instruments have been sterilized in paraffin oil for 10 minutes at 150 C., cooled and, when not in use, kept covered with a sterile towel throughout the operation. The guinea-pigs have been completely immersed from 1½ to 2 minutes in paraffin oil at the temperature noted, drained for a moment and placed on a thoroughly flamed necropsy board. Sometimes, particularly during necropsy of our controls, a sterile towel also has been laid on the board. Separate instruments have been used in the handling of each organ. The pleural and abdominal cavities have been opened separately to avoid the spread of blood. The assistance of an aide and rapid work greatly minimize the opportunities for contamination. In spite of the most rigorous precautions, some contaminations probably occurred from the air or were originally present in the organs of the animal.

TABLE 1

RESULTS OF FIRST EXPERIMENTS, PERFORMED WITH STRAIN 97, TYPE A—MANY RODS
Toxic Spore Suspension, Subcutaneous

No.	Guinea-Pig Weight, Gm.	Dose	Product	Symptoms	Survival	Positive Organs	Remarks
1	300	0.1 c c	Supernatant toxin	Botulism	Died after 45 hrs.		
2	300	0.1 c c	Supernatant toxin heated 1 hr. at 80 F.	Survived	Centrifugalized 1½ hrs.
3	280	1.0 c c	Second wash before heating	Botulism	Died after 21 hrs.	Centrifugalized ½ hr.
4	290	1.0 c c	Second wash after heating	Botulism	Died after 5 days	Spleen, mes. liver, blood bone marrow (mice + +)	Centrifugalized ½ hr.
5	280	2.0 c c	Second wash after heating	Botulism	Died after 45 hrs.	Centrifugalized ½ hr.

Spore Injections, Subcutaneous (Dose, 2 c c)

7	244	1,120 B	Botulism	Died after 24 hrs.	Spleen, liver (mice + +)	
8	247	1,120 B	Botulism	Died after 20½ hrs.	Blood Spleen } mice + Liver } Stomach } Bonemarrow } Mes. } mice + Liver } Spleen }	
9	279	560 M	Botulism	Died after 24 hrs.	Liver, spleen blood mouse + +	
10	258	560 M	Botulism	Died after 24 hrs.		
12	330	112 M	Botulism	Died after 4 days		
13	345	112 M	Botulism	Killed after 7 days, moribund	Liver Brain } mice + Ing. } Spleen }	
14	308	11.2 M	Not botulism	Died after 18 days	General infection, gained weight
15	395	11.2 M	None	Survived		
17	320	1,120 M	None	Survived		
18	265	1,120 M	None	Survived		

Fed Spores (Fed While Fasting)

20	310	2,240 B	Botulism 7 days	Killed after 8 days	Blood, spleen, mes., liver	All mouse +
21	410	2,240 B	Botulism 7 days	Killed after 8 days	Liver, spleen, mes., R. bone	All mouse +
22	380	1,120 B	None, lost weight	Killed after 10 days	Lung, bone, liver	All mouse +
23	320	1,120 B	None, lost weight	Killed after 10 days	Mes., bone, spleen, ing., liver	All mouse +
24	250	560 M	None, gained weight	Killed after 15 days	Bone, lung, spleen, ing., liver, feces	Feces mouse +
25	250	560 M	None, gained weight	Killed after 15 days	Ing., feces	Feces mouse +
26	240	280 M	None, gained weight	Killed after 16 days	Inguinal lymph-node	
27	240	280 M	None, gained weight	Killed after 16 days	Liver, lungs	
29	250	140 M	None, gained weight	Survived		
30	280	140 M	None, gained weight	Survived		
31	250	70 M	None, gained weight	Survived		
32	250	70 M	None, gained weight	Survived		
19	350	Check	Lost weight	Died after 19 days	Ing., kidney (mice + +)	Intercurrent infection

Ing., inguinal lymphnode; Mes., mesenteric lymphnode; Bone, bonemarrow; B, billion; M, million.

It is to be borne in mind that the subsequent use of the word contaminated as applied to the culture of organs does not necessarily imply contaminations from without. In cases in which organisms so closely associated culturally and morphologically as *B. botulinus* and *B. sporogenes*, pathogenic and slightly pathogenic, are growing together in the intestinal tract, it is not unreasonable to suppose that the invasion of the organs by the former may carry through certain individuals of the other species. Heller²⁵ mentions that while *B. sporogenes* is not invasive in small doses in pure culture, it may invade the tissues in company with other organisms. This appears to be true for other anaerobes as well. Also since it has been recently stated²⁶ that the gallbladder and its ducts in 63% of human cases brought to operation contained bacteria, it is not surprising occasionally to find invasive organisms other than *B. botulinus* in the organs of our guinea-pigs. Stress is laid on these facts in order that the thoroughness of our technic may not be questioned.

Cultures.—The beef heart and liver agar mediums and the reasons for their use in the botulism work of this laboratory have already been fully discussed by Dubovsky and Meyer.²⁰ As the final determination of the presence of *B. botulinus* in cultures, unless antitoxin neutralization tests are to be made, depends on the heat resistance of its spores, we have found it desirable to use this medium.

The organs were placed in the freshly boiled and cooled beef heart medium and incubated from 7 to 15 days at 35 C. and reincubated when necessary. All cultures, regardless of appearance or lack of odor, were examined microscopically, and material from those tubes which showed sporulating organisms in any way morphologically resembling *B. botulinus* or free spores with or without bacilli, were inoculated into deep agar, heated from 1 to 1½ minutes at 100 C., incubated from 1 to 7 days at 35 C., and the character of the colonies observed. When such colonies or a sufficient number of them were doubtfully positive for *B. botulinus*, the original beef heart culture was inoculated into mice. In many apparently sterile beef heart cultures a long search will result in the finding of an occasional free spore where nothing else is seen. These spores may or may not prove to be viable. In contaminated cultures, more particularly in those in which the contaminating organisms are cocci or anaerobes of the *B. bifermentans* or *B. sporogenes* types, the latter may almost completely overgrow the few organisms or spores of *B. botulinus* originally in the culture, as has been explained by Reddish.²⁷ In this paper, he also mentions the possibility of an agar colony of *B. sporogenes* containing within its body spores of *B. botulinus*. The impossibility of seeing these, as well as the failure to recognize the atypical colonies of *B. botulinus*, may have caused us to give some erroneously negative findings. Considering the foregoing as well as other sources of error, and in spite of the precautions taken, we believe that we have sometimes failed to show the presence of *B. botulinus* spores in the organs of our experimental animals and that the results given in our tables are possibly too low. However, we wish to state that in the organs of our controls every possible method was used to demonstrate the presence of the organism.

Cultures of the following organs were always made: Blood, bone marrow, inguinal and mesenteric lymph nodes, liver and spleen, and often the brain, lungs, kidneys and sometimes the feces and thigh muscle and gallbladder. The results of our experiments are now presented.

²⁵ Jour. Bacteriol., Jan. 1922, 7, p. 29.

²⁶ Beitr. z. klin. Chirurg., 1922, 125, p. 377.

²⁷ Jour. Infect. Dis., 1921, 29, p. 126.

EXPERIMENTAL DATA

Our preliminary experiments (tables 1 and 2) do not conform in all respects to the description of our technic, but as the results have an important bearing on the subject to be discussed later, we have decided to include them. The spore suspension in the first experiment and the heated and unheated toxin and final wash water (table 1) were ready too late on a Saturday afternoon to inject and so were left in the icebox until the following Monday morning (about 40 hours), when they were injected. They proved to be toxic.

TABLE 2

RESULTS OF EXPERIMENTS WITH STRAIN 38, TYPE A—FEW VEGETATIVE FORMS
Toxic Spore Emulsion: Final Heated Wash Water (Icebox 48 Hrs.) Subcutaneous

No.	Guinea-Pig Weight, Gm.	Dose	Symptoms	Survival	Positive Organs	Remarks
52	178	2 c c	Botulism	Died after 9½ days	All but brain	
53	170	2 c c	Botulism	Died after 4 days	Liver, spleen	
54	158	1 c c	Botulism	Died after 12 days		
55	150	1 c c	Botulism	Died after 11 days		

Subcutaneous Spore Injections

56	294	1.8 B	Botulism	Died after 20 hrs.	All + but ing. and brain	
57	248	1.8 B	Botulism	Died after 5½ days	Brain, liver, bone, mes., blood	
58	255	900 M	Died in night	Died after 18 hrs.	Ing., blood, mes., spleen bone	
59	240	900 M	Botulism	Killed after 3 days	Mes., spleen, liver, bone	
60	199	180 M	Botulism	Died after 14 days		
61	222	180 M	None	Survived		
62	230	Cheek	None	Killed after 16 days	Bone (mouse +) all other organs negative	
63	205	18 M	Indefinite	Died after 12 days	Intercurrent infection
64	202	18 M	Indefinite	Died after 13 days	Intercurrent infection
65	217	1.8 M	Indefinite	Died after 7 days	Intercurrent infection
66	203	1.8 M	Indefinite	Survived		

Fed Spores (Fed While Fasting)

67	220	3.3 B	Botulism	Died after 4½ days	Ing., liver, stomach	
68	300	3.3 B	None	Killed after 10 days	Ing., liver	
69	200	1,650 B	None	Survived		
70	190	1,650 B	None	Survived		
72	165	1,250 B	None	Survived		
73	183	1,250 B	None	Survived		
74	181	725 M	None	Survived		
75	222	725 M	None	Survived		
	240	Cheek	None	Killed after 16 days	All organs negative	

As these results were so at variance with those of previous workers, even though the wash water containing few spores and consequently the spore suspension (afterward diluted) were toxic, we decided to repeat this experiment under similar conditions with a portion of the emulsion used in the experiment summarized in table 3.

In this duplicated experiment, the heated spore suspension and final heated wash water were left in the icebox for 48 hours (Table 2).

These two experiments show the necessity of injecting spore suspensions immediately after heating.

The result of our next experiment, in which the spores were completely detoxified, is given in table 3.

As will be seen from a comparison of the data given in the foregoing tables, an enormous number of *B. botulinus* spores are necessary to produce symptoms and death, especially when fed to guinea-pigs.

As we were able to cause symptoms or death in our guinea-pigs only by the injection and feeding of spores of *B. botulinus* in doses considerably in excess of those employed by other investigators, and as we found a similar irregularity in our results, we thought it would be of interest to ascertain whether it were possible to break the apparent immunity of some of our animals to large doses. Several of these guinea-pigs had survived without presenting symptoms, while others receiving the same dose succumbed.

It has long been known that the spores of pathogenic bacteria, when freed from their toxin, may remain latent in the tissues of the body for considerable periods, and when anything happens, such as slight localized infections or prophylactic vaccinations, to upset the equilibrium of the bodily defenses or to break down its resistance, such spores may germinate and cause infection. An intensive study of this question has been undertaken by different investigators.

Koser and McClelland²⁸ have shown that while it is unusual to recover the spores of the aerobes away from the site of inoculation, the spores of the anaerobes may be recovered from the different organs up to 4 months after inoculation. They confirm the results of many others, especially as regards *B. tetani*, which has been found in most of the organs of the body in fatal human cases, as well as in experimental animals. Canfora²⁹ recovered free spores of *B. tetani* from the blood stream as early as 12 hours and up to the seventieth day after subcutaneous injection. Vaillard and Vincent,³⁰ working with lactic acid and *B. prodigiosus*, Massart and Bordet,³¹ with lactic acid,

²⁸ Jour. Med. Res., 1917, 37, p. 259.

²⁹ Centralbl. f. Bacteriol., I, O., 1908, 45, 495.

³⁰ Ann. de l'Inst. Pasteur, 1891, 5, p. 1.

³¹ Ibid., 1891, 5, p. 417.

Simple,³² with quinine, and Teale and Bach,³³ with lactic acid, sodium bicarbonate, calcium chloride and other substances, have all been able, under varying conditions, to break down the resistance of the host to the spores of *B. tetani* or of other bacteria by the injection of these substances. The latter workers believe from their experiments that, while sometimes the spores

TABLE 3
RESULTS OF EXPERIMENTS WITH STRAIN 38, TYPE A — FEW VEGETATIVE FORMS
Nontoxic Spore Emulsions; Injected Immediately After Heating

No.	Guinea-Pig Weight, Gm.	Dose	Product	Symptoms	Survival	Positive Organs	Remarks
34	410	0.1 c c	Supernatant toxin	Botulism	Died after 24 hrs.		
35	360	1.0 c c	Supernatant toxin 1 hr. at 80 C.	None	Survived		
36	357	1.0 c c	Second water before heating	Botulism	Died after 24 hrs.		
37	245	1.0 c c	Second water after heating	None	Survived		
39	300	1.0 c c	Second water after heating	None	Survived		
40	278	1.0 c c	Second water after heating	None	Died after 14 days	Intercurrent infection
41	270	2.0 c c	Second water after heating	None	Survived		
42	270	2.0 c c	Second water after heating	None	Survived		
43	250	2.0 c c	Second water after heating	None	Survived		

Fed Spores (Fed While Fasting)

45	275	11 B	Botulism 60 hrs.	Died after 96 hrs.	All + except ing.	Brain not cultured Lung, kidney not cultured
46	272	11 B	Botulism 22 hrs.	Died after 60 hrs.	All +	
47	242	5.5 B	Botulism 60 hrs.	Killed after 71 hrs., moribund	Liver, kidney, bone, brain, blood, mes.	
48	210	2.75 B	None	Survived		
49	229	5.5 B	None	Killed after 14 days	Ing.	
50	192	2.75 B	None, lost 40 gm.	Died after 14 days	All + except lungs	
C	350	Cheek	None	Survived		

do not germinate, at other times they do, but that they do not appear to multiply in the tissues. In many of the different experiments cited in the foregoing, cultures of the organs gave positive findings for the spores of the species of organism injected. Tulloch³⁴ injected 1,000,000,000 toxin-free spores

³² Scient. Mem. Officers and San. Dept. Gov't. India, 1911, No. 43, new series (cited by Koser and McClelland).

³³ Jour. Path., 1920, 23, p. 315.

³⁴ Brit. Med. Jour., 1918, 1, p. 614.

of *B. tetani* into animals without producing the corresponding disease. The subsequent inoculation of *B. welchii* toxin caused the germination of the spores of *B. tetani*. The experimental data summarized in tables 1 and 2 tend to confirm to a certain extent the work of Tulloch as to the accessory action of a toxin, in this case homologous, more especially when the toxic spores were subcutaneously administered. As we were able to produce botulism by feeding toxic spores only in excess of 1,120,000,000 in one case and in excess of 1,650,000,000 in another, it is possible that, due to various factors, absorption of a slight amount of toxin from the intestinal tract is prevented. This phase of the question will be referred to later. When the results of these tables are compared with those recording our experiments with non-toxic spores (table 3) and those to be given later, the differences between our results, especially in subcutaneous injections with toxic and toxin-free spores and those of other workers is apparent. The observations of Geiger, Dickson and Meyer¹⁰ as to the difficulty of detoxifying spores by washing or slight heating at 80 C., as many of these investigators have done, are borne out, and this may be one of the causes of the discrepancies between their results and ours. The cultural findings in guinea-pig 52 (table 2) which received toxic wash water subcutaneously is interesting. While the injection of this water would probably have caused a fatal outcome in this case had it not contained very few spores, its toxicity would appear to have been instrumental in causing the spread of these spores, or the bacilli possibly arising from them, throughout all the organs of the body except the brain. This does not seem to occur even in subcutaneous injection unless many millions of detoxified spores are introduced. Here, again, we probably have a confirmation of Tulloch's findings.

Bullock and Cramer⁶ and Edmondson, Giltner and Thom⁵ have shown that calcium chloride injected at the same time or within a few minutes after the subcutaneous injection of pathogenic spores, the latter working with *B. botulinus*, greatly diminishes the time necessary to produce symptoms or death. The latter workers showed that such injections produced no effect if given two days after the spore injection. Their animals were all injected subcutaneously both times.

Though Teale and Bach³³ had fewer failures by the intraperitoneal route, our injections were generally made subcutaneously within 1 to 2 cm. of the site of the original injection. Proper controls for each substance were also made. In view of the results of the latter workers, we are not altogether surprised at our failure by this means, after the interval shown in our tables, to alter the effect of the spores when either fed or injected. Our cultural studies of the organs of other animals lead us to suppose that the tissues of the guinea-pigs thus injected had already been invaded by the spores or bacilli at the time of the injection of such additional substances, and any negative chemotactic property these substances may have possessed was useless at the time they were injected, so that this failure must be attributed to a different cause. Tables 4, 5 and 6 show our experiments in detail.

During the course of these experiments, we endeavored to determine how rapidly the spores of *B. botulinus* leave the site of subcutaneous and intraperitoneal injections. We inoculated 2 cc of a suspension of detoxified spores containing a few bacilli, some of which colored partially by the Gram method, into the peritoneum of 2 guinea-pigs, one of which had been previously

TABLE 4
RESULTS OF EXPERIMENTS WITH STRAIN 58, TYPE A—VERY FEW RODS
Subcutaneous Injections

No.	Guinea-Pig Weight, Gm.	Spores	Symptoms	Survival	Organs Positive	Remarks
80	260	504 M	Botulism	Died after 15 days	All organs + spores in liver	
81	275	504 M	None	Killed after 25 days	Spleen, liver, mes., ing.	After 9 days received 0.5 cc N. Na ₂ CO ₃ intraperitoneally, no symptoms
82	254	252 M	None	Died after 15 days	All organs + spores in liver	
83	227	252 M	None	Killed after 21 days	Liver, bone, ing.	After 9 days received 0.5 cc N. Na ₂ CO ₃ intraperitoneally, no symptoms
84	225	42 M	None	Killed after 24 days	Liver, spleen, kidney, bone, mes.	After 8 days received same as above
85	214	42 M	None	Survived	After 14 days received 0.5 cc N. lactic acid intraperitoneally, no symptoms
86	214	Check	None	Survived		

Fed Spores (Fed While Fasting)

87	223	Check	None	Survived	Neeropsy, table 6
88	300	2.520 B	None	Killed after 10 days, gained 60 gm.	Liver, brain, L. bone	
89	296	2.520 B	Typical botulism 7 days	Killed after 8 days, moribund	Lungs	
90	288	1.008 B	None	Killed after 24 days	Ing., feces	
91	280	1.008 B	None	Survived	After 13 days fed 0.00675 gm. quinin sulphate, no symptoms
92	259	504 M	None	Survived		
93	258	504 M	None	Killed after 27 days, gained 118 gm.	Bone, feces +	
94	251	252 M	None	Killed after 24 days, gained 92 gm.	All negative, feces +	
95	245	252 M	None	Survived	After 9 days received 0.5 cc N. lactic acid intraperitoneally, no symptoms
96	243	Cheek	None	Survived	All negative	
97	242	1.176 B	None	Killed after 21 days	Liver	After 14 days received 0.005 gm. quinin sulphate subcutaneously, no symptoms

injected in the same manner with 5 cc of sterile broth. At 15 minute intervals after injection of the spores, a few drops of fluid were withdrawn from each animal and stained by the Gram method on a slide containing a smear from the spore suspension inoculated. No microscopic differences were apparent in preparations from the 2 guinea-pigs. The leukocytes englobed the

spores and bacilli, and at the end of 6 hours they had practically disappeared from the site of inoculation. Three guinea-pigs were injected subcutaneously with 2 cc of an emulsion of detoxified spores, a pocket having been made under the skin with the needle of the syringe. One guinea-pig also received mixed with the spores, 1 cc of a 2% solution of calcium chloride and another, 1 cc of *B. botulinus* antitoxin added to the spores. The death of the guinea-pig receiving calcium chloride preceded that of the control by a long period of time. Again no differences were noted in the stained preparations after varying intervals up to 24 hours. In the beginning, few leukocytes were present, but they gradually appeared until at the end of 24 hours an intense leukocytosis was present. The disappearance of the spores and bacilli was rapid, and at the end of 5¼ hours few were microscopically visible, either free or in the leukocytes. A few rare leukocytes at this time were gorged with spores and bacilli both in the guinea-pig receiving antitoxin and in the one receiving calcium chloride. Saline washings from these pockets were nontoxic for mice. While it is more than likely that some of the injected spores germinated in these pockets or in the peritoneum within 24 hours, we have no reliable evidence that this was the case. There were not sufficient differences between the Gram stained preparations from the animal fluids and the original spore material on the same slide to warrant a definite statement that this is so. We have sometimes noted a few gram-positive organisms in our heated spore suspension, so that unless there is marked growth or additional evidence is obtained that these rare bacilli seen within a short time after injection are *B. botulinus*, one should accept with reserve these findings as evidence of growth. The result with calcium chloride is not in accord with that of Thom, Edmondson and Giltner,⁶ who found that the leukocytes did not englobe the bacilli and presumably also the spores of *B. botulinus* when injected with this substance. These authors remark that smears showed a few bacilli in the exudate from these subcutaneously injected guinea-pigs and that "cultures revealed the presence of the bacilli in virulent form," but they do not state how they determined that their cultures originated from bacilli rather than from spores. They suggest from the presence of bacilli on their smears that growth of *B. botulinus* must have occurred in the animal body, but they do not mention any controls of colorations of the spore material injected, nor do they state the method used to color their smears nor describe the bacilli seen. Their results are interesting but need amplification.

Teale and Bach³³ have stated that they failed to prevent the phagocytosis of bacteria by injecting lactic acid simultaneously with the bacteria. These authors discuss at length the question of latency of anaerobic organisms and believe that the spores germinate and the vegetative forms thus arising remain latent. Whatever may be the mode of action of these accessory factors in accelerating the appearance of *B. botulinus* symptoms such action would not seem to be due entirely to any negative chemotactic property which such substances may possess for the leukocytes or other phagocytes.

Several of the authors previously quoted have considered that the pathogenicity of the detoxified spores and killed vegetative forms might be due to the action of the enzyme of the blood or tissues in liberating whatever preformed toxin they might contain. While this possibility, for various reasons, seems to us exceedingly remote, we thought it worth while to try the following experiment: Emulsions of counted spores of 2 strains and 1 other in which the number of spores was unknown were heated in sealed tubes at 122 to 125 C. for 5 minutes. The tube in which the spores were uncounted

TABLE 5

RESULTS OF EXPERIMENTS WITH STRAIN 97, TYPE A—MODERATE NUMBER RODS;
FEEDING EXPERIMENTS

Guinea-Pigs

No.	Weight	Product	Symptoms	Survival	Positive Organs	Remarks
98	450	5.08 B + ½ c c N. serum sub- cutane- ously	None, lost 66 gm.	Killed after 30 days	Lungs, brain, feces	After 10 days received 0.005 gm. quinin sulphate sub- cutaneously, no symp- toms
99	420	5.08 B + 1 c c anti- toxin A subcuta- neously	None, gain- ed 10 gm.	Killed after 30 days	Lungs	
100	410	5.08 B + 1 c c N. serum sub- cutane- ously	Not botu- lism, lost 120 gm.	Died after 5 days	Liver	Paratyphoid
101	354	5.08 B + 1 c c anti- toxin A subcuta- neously	Not botu- lism, lost 29 gm.	Died after 3 days	Brain, liver, L. bone	General infection
102	259	Check	Not botu- lism	Died after 13 days	All organs negative	

Mice

1	Medium size	1.27 B + ½ c c N. horse serum subcuta- neously	None	Killed after 10 days	No cultures	
2	Medium size	1.27 B + ½ c c anti- toxin A subcuta- neously	None	Killed after 10 days	Liver, spleen, bone	
3	Medium size	1.27 B + ½ c c N. serum subcuta- neously	None	Killed after 10 days	Blood, bone	
4	Medium size	1.27 B + ½ c c anti- toxin A subcuta- neously	Indefinite	Died after 2½ days	Liver, spleen	
5	Medium size	Check	Killed after 10 days	All negative	

Rabbits

	2,600	63.5 B	None until 31st day dysentery, gained 200 gm. to 20th day	Killed after 35 days	Bone, ing., feces	After 31 days, fed 25,000 M.L.D. filtered toxin; no symptoms except contin- uation of dysentery
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and which contained enormous numbers was immediately opened, 3 drops inoculated into beef heart and the remainder (about 2 cc) injected into a guinea-pig. The other 2 tubes, which contained 17,000,000,000 and 20,000,000,000 spores, respectively, were placed in the incubator and icebox alternately for several hours during 6 days. They were then opened, inoculations made into beef heart and the remaining contents centrifugalized. The deposit and super-

TABLE 6
RESULTS OF EXPERIMENTS WITH STRAIN 62, TYPE A—RARE VEGETATING FORMS
Subcutaneous Injections

No.	Weight	Dose	Symptoms	Survival	Organs Positive	Remarks
109	252	1.459 B	Botulism	Killed after 4 days, moribund	Mes., liver	
110	250	1.459 B	Botulism	Died after 24 hrs.	All organs +	
111	247	230 M	None	Killed after 43 hrs.	Liver, spleen, ing., bone, mes.	
112	232	230 M	None	Killed after 20 days	Ing. (mouse +)	After 23½ hrs. received 0.9 cc 2% N. CaCl ₂ subcutaneously, no symptoms
113	207	230 M	None, 21 days gained 45 gm.	Survived		
114	200	230 M	Botulism	Died after 4½ days	Ing., bone, spleen, liver, brain, blood	After 23½ hrs. received 6.9 cc 2% N. CaCl ₂ subcutaneously, no symptoms
115	285	Check	None	Survived		

Fed Spores (Fed While Fasting)

117	320	11.5 B	Botulism 3 days	Killed after 4½ days moribund	Bone, brain, liver, feces	Lost 73 gm.
118	289	11.5 B	Indefinite	Died after 3½ days	Mes.	Lost 29 gm.
119	286	5.75 B	Botulism 6 days	Died after 13 days	Liver, feces	Lost 126 gm.
120	282	5.75 B	Botulism	Died after 6½ days	Liver, bone, brain	Lost 100 gm.
121	270	2.875 B	None	Survived	Released after 5 wks.
122	270	2.875 B	None	Killed after 15 days	Lungs, brain, feces	Gained 40 gm.
123	260	1.150 B	None	Killed after 15 days	All negative	Gained 52 gm.
124	258	1.150 B	None	Killed after 15 days	All negative	After 22¼ hrs. received 0.9 cc 2% N. CaCl ₂ subcutaneously, no symptoms, gained 79 gm.
125	177	1.150 B	Indefinite	Died after 4 days	Liver, feces	Lost 14 gm.
126	200	1.150 B	Lost 67 gm.	Died after 16 days	All negative, feces +	After 22¼ hrs. received 0.9 cc 2% N. CaCl ₂ subcutaneously, no symptoms
87	330	Check	None	Killed after 16 days	Liver, feces (mice ++)	Gained 50 gm.

natant fluid from each tube (about 1.5 cc) were injected separately under the skin of different guinea-pigs. None of these injections produced symptoms, showing that the protein substance, or its autolyzed products of killed *B. botulinus* spores and rare bacilli in the amounts injected is not toxic when introduced subcutaneously into guinea-pigs. The inoculated mediums from all these suspensions remained sterile.

Though the wall of a viable spore may be highly resistant to heat, we know that when subjected to a temperature of 80 C. for 1 hour the protein contents undergo some damaging changes, if not a certain degree of coagulation, as often evidenced by greatly delayed ability to germinate after being heated at this temperature. Also, it probably could be shown by quantitative cultural studies before and after heating to 80 C., as has been done for a temperature of 100 C., that many of the least resistant spores had been killed at this temperature. That is why we have always checked our microscopic counts by cultivated dilutions after heating. Since the temperature at 80 C. produces some of these vital changes, it is more than likely that it destroys the intracellular toxin of the spores as well.

A summary of the toxicity of the different strains employed in the foregoing experiments, as well as the minimum dose required to produce definite symptoms of botulism is given in table 7.

TABLE 7
TOXICITY OF STRAINS

Strain	Toxic Spore Emulsion	Minimum Dose Given Subcutaneously	Death from Botulism	Minimum Dose Fed	Death from Botulism	Remarks
97	Yes	112 M	4 and 7 days	2.24 B	Killed after 8 days	Very ill; rats killed
97	No	5.08 B	2 survived	1 received antitoxin
97	No	5.08 B	2 died, after 3 and 5 days, botulism very doubtful; 1 received antitoxin
97	No	63.5 B	(Rabbit) survived 14 days,	Killed after 35 days
38	No	2.75 B	1 survived 4½ days,	
38	Yes	180 M	14 days, 1 survived	3.3 B	1 survived 7 days,	
58	No	252 M	15 days, 1 survived	2.5 B	1 survived 13 days,	
62	No	230 M	4½ days, 3 survived	5.75 B	6½ days	

From table 7 it will be seen that strain 58 was the most toxic in the feeding experiments, while strain 38 appears to be the most toxic of those injected subcutaneously. However, due to the few experiments recorded with each strain, the individual susceptibility of the animals or other unknown causes, no definite conclusions can be drawn as to any differences which may exist in the pathogenicity of the spores of each strain. Owing to the fact that many of our guinea-pigs were killed with a view to the study of the organs, perhaps some of these might have developed symptoms had they been allowed to live longer. For this reason, we do not categorically deny the possibility of producing botulism for these strains in doses below those given in table 7, but it would appear that these strains, in our hands, all of which produced culturally a potent toxin, were not pathogenic in small doses like those recorded by other workers for other strains.

A summary of the cultural studies of the various organs of our experimental animals, as shown in the tabulation given in the foregoing, is given in tables 8 and 9. In figuring the percentages of the results of this work, we have included cultures of the organs from a few animals (intraperitoneal and subcutaneous leukocyte tests) that received detoxified spores but which are not included in our tabulated results.

While, as we have said before, we do not believe that we have been successful, especially in our earlier experiments, in demonstrating *B. botulinus* in all of the organs when it has been present, the main fact to be deduced from these results is the invasion of all the organs of

TABLE 8
RESULTS OF CULTURAL STUDIES OF VARIOUS ORGANS OF EXPERIMENTAL ANIMALS

Organ	Subcutaneous						Fed						Total + Died or Dying of Botu- lism
	Died or Dying		% +	Killed, No Symp- toms		% +	Died or Dying		% +	Killed, No Symp- toms		% +	
	+	—		+	—		+	—		+	—		
Ing. lymph.....	5	12	30	3	3	50	4	14	22	4	17	19	25%
Bone.....	11	6	64	2	4	33	8	10	44	8	13	40	54%
Blood (heart)...	9	8	53	0	6	0	2	16	10	0	21	0	31%
Mes. lymph.....	10	7	59	2	4	33	7	11	40	1	20	5	50%
Spleen.....	13	4	77	2	4	33	6	12	33	3	18	14	54%
Liver.....	16	1	94	3	3	50	14	4	80	8	13	38	80%
Kidney.....	5	1	83*	1	0	—	4	9	32*	0	9	0	48%*
Brain.....	6	2	75*	0	4	0	7	6	54*	3	14	12	60%*
Lungs.....	2	1	66*	1	1	—	3	5	37*	8	9	47	45%*
Muscle.....	0	1	0	0	0	0	0	2	0	0	1	0	0
Feces.....	0	0	0	0	0	0	3	0	100*	11	2	91*	100%*
Stomach†													
Total guinea- pigs.....	17			6			18			21			62

* Percentage of times cultures were taken from organ.

† Intraperitoneal 2 guinea-pigs, all + except blood.

the body regardless of the mode of administration of the spores. In those animals in which a subcutaneous injection of spores produced a fatal result, the organism was recovered more often than in those that died after the spores were fed. In the foregoing experiments the few microscopic examinations of smears of these organs, or of the heart blood, which we have made have been negative, with the exception of a few cases in which we have seen rare spores or bacilli in the liver and blood. Undoubtedly enormous numbers of the spores are eliminated by the leukocytes of the blood or by the endothelial cells of the tissues, especially of the liver and spleen.

To what extent the spores and bacilli of *B. botulinus* are actually destroyed and where is problematical. The literature on this subject is filled with interesting discussions based on intravenous injections

into animals of inert substances and bacteria. Drinker and Shaw³⁵ Fenn,³⁶ Nagao³⁷ and numerous others working with manganese dioxide, carbon, India ink, etc., and Nagao³⁸ using streptococci, as well as Meyer, Neilson and Feusier,³⁹ who injected typhoid bacilli, have made a thorough investigation into the fate of these organisms when

TABLE 9
ANIMALS KILLED AFTER SPORE INJECTIONS
Subcutaneous, No Symptoms nor Lost Weight

Days	Liver	Spleen	Bone	Mes.	Ing.	Brain	Kidney	Lungs	Feces
20	+	+	+	+	+
20	+
21	+	..	+	..	+
24	+	+	+	+	+
25	+	+	..	+	+
	4	3	3	3	4	0	1	0	

Fed, No Symptoms Nor Lost Weight

10	+	+	0
10	+	..	+	+
10	+	+	+	0 Mice
10	+	0 Mice
14	+	0
15	+	+	+	+	+
15	+	+
15	+	..	+	+
15	0	0	0	0	0	0	0	0	+
15	0	0	0	0	0	0	0	0	+
16	+	+	0
16	+	+
21	+	0
24	+	+
24	0	0	0	0	0	0	0	0	+
27	+	+
30	+	..	+	+
30	+	..
35	+	..	+	+ Rabbit
	7	2	6	0	5	3	0	5	11

No Symptoms, Lost Weight

10	+	..	+	+	0
10	+	+	+	+	+	0

0 = cultures not taken.

introduced into the blood stream. The work of the latter authors especially shows the important rôle played by the cells of the lungs, liver, spleen, and bonemarrow and the possibility of reinfection of the blood from foci in these organs. It is possible that the irregular findings

³⁵ Jour. Exper. Med., 1921, 33, p. 77.

³⁶ Jour. Gen. Physiol., 1921, 3, p. 465.

³⁷ Jour. Infect. Dis., 1920, 27, p. 527.

³⁸ Ibid., p. 327.

³⁹ Ibid., 1921, 28, p. 408.

in the heart blood of our experimental animals may be due to this cause. Barlett and Ozaki,⁴⁰ who have studied phagocytosis in vivo have shown that the cells of the liver and spleen act to a greater or less extent in a compensatory way for the deficiency of the leukocytes of the blood as they become less capable of ingesting bacteria. They believe this fact to be of great significance in eliminating bacteria from the blood. The fact that we have more often found *B. Botulinus* in these two organs than in the blood in fatal cases might in a measure corroborate this idea. The same result is also obtained in those cases in which latency of the spores occurs or at least in which symptoms from toxin formation are greatly delayed.

It is unfortunate that the brain was studied in only about 50% of our animals, as the findings for that organ cannot be fairly compared with those of a majority of the other organs. However, from the data at hand, the brain tissue in fatal cases seems to play a rôle second in importance only to that of the liver, or perhaps the spores or bacilli find conditions there more favorable for growth than in many of the other organs. The cultures of the bonemarrow and spleen appear to give equal results for all animals dying of botulism, though in the case of fed animals which presented no symptoms, the organism was found more frequently in the bonemarrow than in any other tissue except the lungs. Allowance must be made in the case of the latter organ for possible direct and continuous contamination from feces and food. The persistence of the spores in the feces of our fed animals which survive long periods is interesting. We have seen no evidence which would support the conclusion that toxin is formed in the intestinal tract in quantities sufficient to cause symptoms of botulism when, or if, absorbed into the blood stream.

Though several slightly pathogenic anaerobes of the putrefactive type as well as some of the highly pathogenic saccharolytic anaerobes, notably *B. welchii* and *B. tetani*, lead a semisaprophytic existence in the intestinal tract, the result of the work of this laboratory shows that the spores of *B. botulinus* are rarely found in the feces of normal animals and have not yet been found in the stools of healthy human beings; so that the possibility that the few spores thus encountered or even greatly increased numbers might produce toxin in the intestines is extremely unlikely. Reference to the data presented in tables 1 and 2 will show the enormous doses of spores even when fed in toxic solution that may be required to produce symptoms of botulism as against

⁴⁰ Jour. Med. Res., 1917-18, 37, p. 139.

the number injected subcutaneously which are required to obtain the same results. This would tend to support our contention. It is another matter when toxin-free spores are accidentally or experimentally ingested in prodigious numbers. They are then frequently introduced into an empty stomach in a concentrated mass and possibly are absorbed from points in the tract where they remain concentrated in the fluid and before any great dissemination throughout the tract has taken place. This is possibly a condition which facilitates their absorption, or at least they penetrate the intestinal wall in numbers too great to be destroyed by the natural defenses of the tissues, where the toxin is probably formed or liberated. Bronfenbrenner and Schlesinger⁴¹ theorize as to points in the digestive tract from which the toxin of *B. botulinus* is probably absorbed. They have shown that the potency of the toxin is enormously increased in an acid medium and that in addition to its specificity it contains some substance capable of increasing the permeability of the intestine so as to permit the absorption of the specific toxin. Therefore, they believe that when it encounters the acid medium of the upper digestive tract (stomach and upper duodenum), it is readily absorbed. If that be so, it would seem that any toxin which passes this point or is formed in the lower tract might be neutralized, attenuated, or destroyed by the enzymes or alkaline metabolites produced by other bacteria, and that may be why such enormous numbers of detoxified spores, if they produce toxin at all, are innocuous when ingested.

Having found *B. botulinus* so persistently in the organs of our animals when fed or injected subcutaneously with the spores of this organism, we desired to ascertain if possible which organs were invaded first after the spores reached the blood stream. We, therefore, injected the spores intravenously as well as by other routes into a series of animals and killed them at varying intervals. Unfortunately the dose injected intravenously was so large that our results enumerated in table 10 enable us to form no definite conclusions on this subject.

It is evident that a comparatively small number of spores of *B. botulinus* fed to guinea-pigs do not invade any of the tissues, but the principal information gathered from this experiment proves that the same number of spores, when once in the blood stream, rapidly invade all the tissues of the body. We wish also to call attention to the fact that all the organs of 4 guinea-pigs in this series (2 cage checks and 2 fed) were negative for *B. botulinus*. This, taken in conjunction with

⁴¹ Jour. Am. Med. Assn., 1922, 78, p. 1519.

the cultural studies of the organs of our other check animals, in which we have rarely found *B. botulinus*, should be taken as sufficient evidence that our cultural findings in these experiments are the result of our inoculations of spore suspensions.

Meyer, Neilson and Feusier ³⁹ have shown that the distribution and destruction of small or large doses of typhoid bacilli are practically the same in normal and immunized rabbits; so that the question arose in our minds as to whether the neutralization of the toxin of *B. botulinus*

TABLE 10
RESULTS OF EXPERIMENTS WITH STRAINS 97, TYPE A AND 108, TYPE B MIXED CULTURE;
PRACTICALLY NO RODS

Jugular Vein							
No.	Weight	Spores	Symptoms	Survival, Hours	Killed or Died	Positive Organs	Remarks
131	336	Cheek	None	114	Killed	None	Blood coagulated, little taken
132	350	20 M	None	18	Killed	All	
133	360	20 M	None	43	Killed	All	
134	344	20 M	None	46	Killed	All	
135	350	20 M	Botulism	67	Killed, very ill	All	
136	350	20 M	Botulism	70	Killed, very ill	All	
137	323	20 M	Botulism	60	Died	All	
138	356	20 M	Botulism	80	Died	All	
140	256	20 M	None	17	Killed	All except blood, brain, kidney, mes.	
141	265	20 M	Botulism	69	Killed, very ill	All	
Subcutaneous							
142	309	20 M	None	85	Killed	Liver, spleen, ing.	
143	315	20 M	None	87	Killed	All	
Fed							
144	360	750 M	None	Survived			
145	265	20 M	None	95	Killed	None	
147	230	20 M	None	96	Killed	None	
146	200	Check	None	97	Killed	None	

as formed in these intravenous injections could have any influence on the distribution of this organism in the various tissues. We, therefore, inoculated 4 guinea-pigs with a similar dose of a different strain and 10 minutes later injected subcutaneously 2 of them with normal horse serum and 2 with antitoxin corresponding to the type of organism injected. We also endeavored to determine whether there was circulating toxin in the emulsified organs of any of these guinea-pigs, and, finally, whether the cultures of these organs were produced by spores

(and bacilli) or by bacilli alone. We found that the emulsified brain, spleen and liver of one guinea-pig (155) injected with antitoxin were toxic for mice in 5, 4 and 6 days, respectively, but the same organs of guinea-pig No. 153, receiving no antitoxin, were not toxic for mice. This result is difficult to interpret, and the experiment should be repeated on a larger scale before forming any conclusions. A microscopic study of the emulsions of the organs gave the following results: Rare spores and gram-positive bacilli were seen in preparations of the blood of No. 152, while the blood of No. 154 was negative. The spleen and liver of No. 153 showed rare gram-positive bacilli, resembling *B. botulinus* but no spores, while the same organs of guinea-pig 155 were negative. The blood of these last two animals was not examined microscopically. The other results obtained in this series are shown in table 11.

A study of this table shows, as in our previous experiment with intravenous injections, the rapid dissemination of the organism throughout the body. The failure to recover from the brain in most of these cultures is unusual. It is possible that this organ is the last to be invaded, and under the conditions of this experiment the animals died or were killed (except 155) before the organism had lodged in the brain. In experiments previously reported, we have frequently failed to recover the organism from the brains of animals dying within 48 hours. All heated culture tubes which proved sterile had been incubated from 13 to 15 days. It should be stated that as nearly equally large amounts of emulsion as possible, were inoculated into each tube. The outstanding feature of this experiment is the direct proof of germination of the spores of *B. botulinus* in the animal body, as shown by the failure to obtain cultures from those tubes which were heated, when the same emulsion in unheated tubes gave a positive culture. This occurred 6 times and shows conclusively that these organs had either been invaded by vegetating forms or that the spores which they might have contained had germinated. No great difference is apparent between the cultures of the organs of those guinea-pigs which did and those which did not receive antitoxin.

After this encouraging result, we attempted to demonstrate in an even more conclusive manner the germination and possible multiplication of these organisms in the animal body. A tied loop or pocket was formed in the jugular vein of 3 rabbits. One-half to 1 c c of emulsion containing from 50,000,000 to 250,000,000 freshly detoxified spores was injected with aseptic precautions into this tied vein. After 48 to 69

hours this portion of the vein was excised without disturbing its contents, and a drop of liquid from the vein stained by the Gram method. The vein and contents from 2 rabbits was ground up with sterile saline and injected into mice with fatal results in 6½ and 17 hours. Microscopic preparations of this emulsion, as well as from the contents of the

TABLE 11
RESULTS OF EXPERIMENTS WITH STRAIN 23, TYPE A—(FROM BROTH) RARE GRAM-
NEGATIVE RODS
Serum Injections Subcutaneous

Intravenous					Positive Organ Emulsions				
No.	Weight	Spores	Symptoms	Survival		No. 152	No. 153	No. 154	No. 155
152	320	15 M + 0.5 c c N. serum	Botulism	Died after 45 hrs	Lungs {Heated	+	+	0	0
					Unheated	+	+	+	+
153	369	15 M + 0.5 c c N. serum	Botulism	Died after 70 hrs.	Kidney {Heated	—	—	0	0
					Unheated	+	+	+	—
154	388	15 M + 0.5 c c anti- toxin A	None	Killed after 47 hrs.	Mes. {Heated	+	+	0	0
					Unheated	+	+	+	+
155	389	15 M + 0.5 c c anti- toxin A	None	Killed after 69 hrs.	Ing. {Heated	—	+	0	0
					Unheated	+	+	+	+
					Bone {Heated	+	+	0	0
					Unheated	+	+	+	+
					Spleen {Heated	+	+	+	+
					Unheated	+	+	+	+
					Liver {Heated	+	+	+	+
156	424	225 M	Botulism	Died after 6 days	Unheated	+	+	+	+
157	470	225 M	Botulism	Died after 7 days	Brain {Heated	—	—	—	—
					Unheated	—	0	—	+
					Blood {Heated	—	—	+	0
					Unheated	—*	+	+	+
					Gall {Heated	—	—	—	0
					Unheated	—	—	—	+

* Small amount inoculated; 0, cultures not taken.

vein alone from all rabbits showed rare spores and a definite growth of gram-positive organisms with division (7 to 10 per microscopic field) which morphologically resembled *B. botulinus*. These emulsions immediately inoculated in equal amounts into deep agar (heated and unheated) gave a luxurious growth of typical colonies of *B. botulinus* in the unheated tubes, while those that were heated showed only rare

colonies of the same organism. The rabbits died in from 3½ to 6 days after injection. The centrifugalized blood serum of all the rabbits was fatal to mice. The emulsion of bone marrow and spleen of 2 rabbits were tested and only the spleen of 1 rabbit proved sufficiently toxic to produce death in a mouse in 36 hours. We also attempted to produce a growth from the spores of *B. botulinus* in the anterior chamber of the eyes of 2 rabbits. The fluid from the eye of 1 rabbit, removed after 76 hours, showed only a few gram-positive bacilli with division, but no spores. The fluid from the eye of the other rabbit, at the end of 20 hours, while most of the spores and bacilli had been removed by the intense leukocytosis which prevailed, thus duplicating the phagocytosis in the peritoneal fluid, enough gram-positive bacilli with end to end forms remained to show a definite germination and multiplication of the *B. botulinus* spores injected. Inoculation of this fluid directly into agar gave a culture of *B. botulinus* contaminated only by a few colonies of cocci. The fluid from the eye of each rabbit was fatal for mice (table 12).

We consider the results of these last two experiments as giving definite and conclusive evidence that the detoxified spores of *B. botulinus* actually germinate and multiply in the animal body. To just what extent this multiplication continues, we are not prepared to express an opinion. In the rabbits intravenously injected, death may have been caused by the transudation of the toxin through the vein wall into the circulating blood, or as the tied vein was not completely stripped of its minute collateral branches some of the bacteria or toxin may have escaped into the blood stream through these small vessels and produced more toxin there. In the same way, it is possible to explain positive cultures for some of the organs. The culture of these spores in a tied vein simulates closely the culture *in vitro*. The circulation of the blood is prevented or is exceedingly sluggish and more nearly resembles that of the lymphatic and retarded peripheral circulation of the blood which may be favorable to the growth of bacteria. In all events the growth, especially in No. 3, was most conclusive.

DISCUSSION

Whether a few spores remaining latent in the intestinal tract could ever find conditions favorable to extensive growth and consequent toxin production or invasion of the tissues seems extremely unlikely and remains for further quantitative cultural studies to decide. From the

argument presented in considering the results of our cultural studies, it would appear that unless introduced in enormous numbers we have little to fear from the ingestion of toxin-free spores of *B. botulinus*, but we have shown that, when thus introduced, they cause symptoms and death from botulism. It has not yet been proved that the ingestion of small quantities of spores together with sublethal doses of toxin

TABLE 12
RESULTS OF EXPERIMENTS WITH STRAIN 23—RARE VEGETATIVE FORMS; INJECTION OF
HEATED SPORE SUSPENSION
Tied Vein of Rabbits

No.	Dose, C c	Amount	Vein Ex-cised	Microscopic Vein Emulsion	Tox-icity Vein	Sur-vival, Days	Toxicity Organs for Mice	Cultures Organs
1	0.5	Approx. 50 M	69 hrs.	Rare spores, rare gram + bacilli division	6	Blood serum +	Liver, blood neg.
2	0.75	Approx. 100 M	48 hrs.	Rare spores, rare gram + bacilli division	+ typical 17 hrs.	4	Blood serum + Bone, spleen —	Blood neg. Spleen, bone +
3	1.0	Approx. 250 M	66 hrs. agar culture heated + unheated + + + +	Rare spores, good growth, gram + bacilli division	+ typical 6½ hrs.	3½	Blood + Bone — Spleen + 36 hrs.	Blood neg. Bone, heated — unheated + Spleen, heated + unheated +

Anterior Chamber of Eye

4	..	Not counted	Fluid withdrawn 76 hrs.	No spores, rare gram + bacilli division	Eye fluid + 24 hrs.	7	Blood serum +	Eye fluid + + Bone — Spleen, heated — unheated + Blood —
5	..	Not counted	20 hrs. agar culture heated + unheated + + + +	Several spores, many gram + bacilli division	+ 28 hrs	2	Blood serum —	Eye fluid + Bone + Spleen + Liver + Blood —

can produce symptoms or death from botulism, but from our few experiments with toxic wash water containing few spores, taken in conjunction with the experimental evidence obtained by other workers³⁴ along these lines, it is quite likely that under these conditions they may invade the tissues, proliferate and produce enough additional toxin to cause symptoms of the disease. The fact that the organs of fatally infected animals are generally microscopically sterile is partial evidence that the toxin is produced from exceedingly few vegetative forms.

When we consider the potency of the toxin of *B. botulinus*, this need not surprise us. It is probable that the spores do not find conditions equally favorable for germination in all the tissues of the body and that the vegetative forms which arise from the few spores which survive the enzymes or phagocytes are, in turn, quickly destroyed and their toxin liberated in the process. This may be why we have been able to recover the organisms in some cases from so few of the tissues of animals which have succumbed to spore injections.

We have no theory to offer as to delayed infection by latent spores for in none of our experiments has this occurred. Neither have we, by the usual procedures for other bacterial species, been able to produce such an infection after several days in animals which had shown no symptoms. Undoubtedly the spores or bacilli of *B. botulinus*, possibly in small numbers, remained latent, particularly in the liver and bonemarrow, for considerable periods after the animals had received massive doses. Whether these latent organisms, after once being established in equilibrium with the fluids and tissues of the body ever germinate or multiply later in sufficient numbers to produce the disease, is more or less of an open question. We are aware that botulism has occurred in animals which had been fed several months previously with spores and maintained afterward in cages grossly contaminated with dejecta or foods containing spores of *B. botulinus*, but we cannot be sure in these rare cases that there may not have been a continuous reinfection of the lungs and intestinal tract from the feces or food remaining in the cage. We have seen 1 normal animal in such a cage die of symptoms suspiciously resembling those of botulism.

Why some animals should succumb to botulism and others of the same weight receiving the same large dose under identical conditions should fail to show symptoms is another question which we have not attempted to answer. A natural immunity probably does not exist except under the conditions of the experiments, for we have later killed some of these surviving guinea-pigs with small doses of toxin. Neither in this case is it a question of the toxin having caused the germination or multiplication of latent spores or bacilli, for the normal controls died within the same period.

The mode of action or the paths of diffusion of the toxin of *B. botulinus* in the animal body has received no attention in our experiments, so that we have not sufficient information to discuss this phase of

the question. Nor are we able to venture an opinion as to which of the tissues may play a preponderating rôle in the neutralization of the toxin as produced. The fact that emulsions of the organs of animals dying of botulism are so infrequently toxic leads us to suppose that the amount of toxin circulating in the system, at least postmortem, is very minute. It may be that at the time that the symptoms were produced and the damage done to the system it was in greater quantity and had been subsequently attenuated or destroyed by fixation in the tissues, or the process of this fixation may have been the condition causing the manifestation of symptoms.

While it is remotely possible, though we have no evidence to support the belief, that some of the toxin liberated in the animal body may be derived from a preformed endotoxin within the heated spore or bacterial cell, we have demonstrated beyond doubt that the toxin-free spores of *B. botulinus* do germinate and multiply in the fluids and tissues of the animal body and produce toxin there. Our cultural studies would tend to substantiate these conclusions. Therefore, in our opinion, the principal, if not the only, source of toxin in the body of our experimental animals is from the germination of these spores.

We wish to say in conclusion that our results showing the difficulty of infecting laboratory animals except by massive doses of heated *B. botulinus* spores should not be taken in any way as a criterion for what may happen in the human system by the consumption of spores of this organism in the natural way—although undoubtedly in the latter case enormous numbers if freed of their toxin would be required to produce infection.

CONCLUSIONS

Massive doses of toxin-free spores of *B. botulinus* are pathogenic when introduced into the animal body.

These spores and the vegetative forms arising from them are rapidly disseminated throughout the tissues of the body.

Toxin-free spores of *B. botulinus* germinate, and the vegetative forms arising from this germination multiply and liberate toxin in the animal body.

THE HEAT RESISTANCE OF THE SPORES OF B. BOTULINUS AND ALLIED ANAEROBES. XI

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As a part of a broad study dealing with the bacteriology and biochemistry of *B. botulinus*, the heat resistance of the spores of this and allied anaerobes has been investigated. A brief analysis of the accumulated data made on 1804 spore suspensions and 65 soil specimens naturally or artificially contaminated with the spores of *B. botulinus*, has revealed a number of important scientific and practical facts. As the detailed preparation and publication of the numerous experiments embracing over 42,807 cultures may be delayed for several months, it is deemed advisable to report the main tentative conclusions. The technic used in these studies is in principle identical with that employed by Bigelow and Esty¹ in their work on the heat resistance of thermophilic bacteria. In the course of the investigation minor modifications in the preparation of the cultures and the subcultures of the heated spore suspensions have been adopted in order to eliminate the danger of laboratory contaminations. Extensive experience has definitely proved the broad applicability and absolute reliability of this standard method.

COMPARATIVE RESISTANCE OF SPORULATING ANAEROBES TO MOIST HEAT

(a) *B. botulinus*.—A comparative study of 109 strains of *B. botulinus*, which originated from numerous field specimens, soils and vegetables as well as food products responsible for 26 human and 9 chicken outbreaks of botulism, reveals two facts: (1) certain strains sporulate irregularly and poorly, and (2) the heat resistance fluctuates within wide limits. The spores employed in these tests have been produced in canned pea infusion—peptic-digest broth (equal parts P_H 8.4). After an incubation of 10 days at 35 C. the culture has been concentrated by centrifugalization and the sediment suspended in 50 c.c. of a M/15 phosphate solution of a P_H of 7.00 to 7.12 (Sorensen mixture of Na_2HPO_4 and KH_2PO_4). The mixture has been vigorously shaken in order to produce a uniform suspension, and 2 c.c. amounts have been delivered with the aid of a sterile buret into tubes of hard glass with a uniform bore, thickness, etc. These tubes have been sealed in an oxygen blast and heated at 105 C. (221 F.) in a DeKhotinsky electric oil bath. The temperature has been controlled by means of a thermo-regulator and kept uniformly distributed throughout

Received for publication, July 26, 1922.

¹ Jour. Infect. Dis., 1920, 27, p. 602.

the bath by a turbine stirrer device. Subcultures of the heated spore suspensions have been made in a 0.5% glucose-beef-heart-peptic-digest broth (PH 7.0-7.4) stratified with vaseline and incubated at 35 to 37 C.

The 109 strains are divided into 3 groups according to their toxicologic and serologic characteristics: type A, 78; type B, 30; nontoxic, 1 strain. Their relative heat resistance is shown in table 1.

At a temperature of 105 C. (221 F.), the average survival time of the spores of 78 strains of *B. botulinus*, type A, is 40.1 minutes, while the minimum is less than 9 minutes and the maximum 85 minutes. The survival time of the spores of 30 type B strains at the same temperature is on the average 23.7 minutes, with a minimum of less than 6 minutes and a maximum of 60 minutes. The average resistance of the 109 strains of *B. botulinus* is 35.2 minutes at 105 C. (221 F.). Spores of type B are apparently less heat resistant than those of type A strains. It is, however, evident that under exceptional circumstances strains and spores of a resistance equal to that recorded for type A strains may be encountered in nature or may be produced artificially in the laboratory.

TABLE 1
RELATIVE HEAT RESISTANCE OF THREE GROUPS OF *B. BOTULINUS* STRAINS

Resistance at 105 C. (221 F.) in Minutes	Number of Type A Strains	Number of Spores Heated	Number of Type B Strains	Number of Spores Heated	Total Number of Type A and B Strains
Less than 12.....	3	{1 no count 0 12 = 10 M.* and 100 M.	10	{1 no count 0 100 M. to 1 B.	13
12 to 21.....	9	250 M. to 1,300 B.	5	200 M. to 3 B.	14
21 to 42.....	33†	25 M. to 3 B.	7	130 M. to 1,200 M.	40
42 to 60.....	16	130 M. to 3 B.	4	100 M. to 200 M.	20
60 to 90.....	18	250 M. to 12 B.	4	75 M. to 535 M.	22
Less than 12 to 90	79†	10 M. to 12 B.	30	75 M. to 3 B.	109

* M. = million; B. = billion.

† The spores of the nontoxic strain survived 30 minutes but were killed in 33 minutes. Two strains (1 A and 1 B) failed to produce spores either in pea peptic digest broth or brain medium; 9 strains (4 A and 5 B) were grown in minced sheep brain medium with a natural reaction after it was noted that pea peptic digest broth failed to yield a sufficient number of spores to make comparative tests. Spores of 4 strains cultivated and tested in this manner were destroyed in less than 12 minutes.

(b) *B. sporogenes*.—The heat resistance of 33 strains of *B. sporogenes* has been determined at 100, 105 and 110 C. The spores have been produced either in pea peptic digest, veal infusion-peptic-digest-gelatine or brain medium. The following heat resistances have been recorded:

100 C. (212 F.) 10 to 150 minutes; average survival time 72.5 minutes.

105 C. (221 F.) 4 to 45 minutes; average survival time 12 minutes.

110 C. (230 F.) 1 to 12 minutes; average survival time 3.3 minutes.

The cultures of heated spore suspensions exhibit the property of retarded germination. In several instances it has been noted that cultures failed to grow until after 100 days of incubation at 35 C.

(c) *B. tetani*.—The spores of 24 strains of *B. tetani* purified and described by H. H. Heller,² produced either in pea peptic digest broth, brain medium, double strength veal infusion-peptic-digest-gelatine or casein broth, exhibited the following heat resistance:

² Ibid., 1922, 31, p. 31.

100 C. (212 F.) 15 to 90 minutes; average survival time 25 minutes.

105 C. (221 F.) 3 to 25 minutes; average survival time 9.2 minutes.

The maximum resistance was obtained in neutral brain medium. Germination of the heated spores in an optimum medium was not markedly retarded. All surviving spore suspensions showed growth within 6 days.

(d) *B. bifermentans*.—Three of the 4 strains of *B. bifermentans* (isolated and described by I. C. Hall³) survived 30 minutes, but were destroyed in 45 minutes at 100 C. The spores of one strain exposed to a temperature of 105 C. (221 F.) survived 18 minutes, but did not resist 21 minutes.

(e) *B. centrosporogenes*.—Three of 4 strains of *B. centrosporogenes* (isolated and described by I. C. Hall³) were destroyed in less than 30 minutes at 100 C. The fourth strain resisted 30 minutes but not 45 minutes at the same temperature.

(f) *B. welchii*.—One strain of *B. welchii* isolated from a heated soil specimen

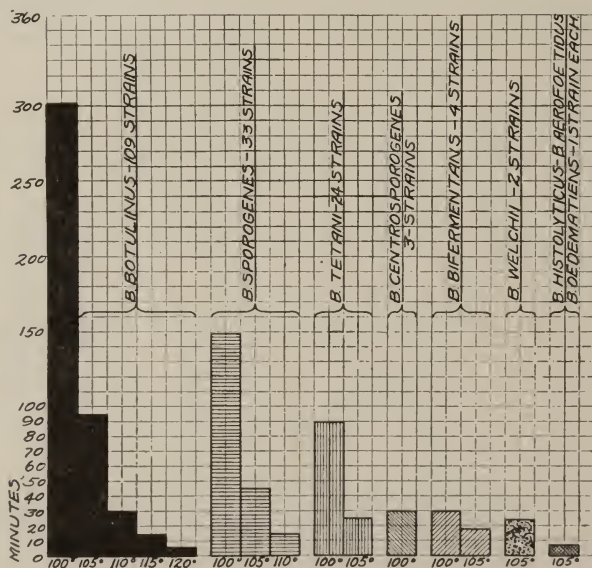


Chart 1.—Maximum heat resistance of sporulating anaerobes.

produced spores in sheep brain medium, which survived 24 minutes but not 27 minutes at 105 C. The spores of another strain were killed in 10 minutes but survived 5 minutes at 100 C.

(g) *B. histolyticus*, *B. oedematiens* and *B. aerofectidus*.—One strain each of *B. histolyticus*, *B. oedematiens* and *B. aerofectidus* failed to survive 6 minutes at 105 C. The anaerobe isolated by Bengtson⁴ from the larvae of *Lucilia Caesar* was destroyed in less than 15 minutes at 100 C. The subcultures of the anaerobes mentioned under d, e, f and g exhibited no retarded germination.

The maximum heat resistances of the various anaerobes tested at different temperatures are shown in chart 1. It is definitely demonstrated that *B. botulinus* produces spores of extraordinary resistance toward moist heat.

³ Ibid., 1922, 30, p. 445.

⁴ Pub. Health Rept., 1922, 37, p. 164.

Furthermore, a comparison of these data with those published by von Hibler,⁵ Becker⁶ and others indicates that the resistance of the spores of American strains of *B. botulinus* surpasses that of the sporulating anaerobes thus far investigated.

THE MAXIMUM RESISTANCE OF *B. BOTULINUS*

The maximum resistance to moist heat of *B. botulinus* spores artificially produced under optimum conditions of temperature, food, oxygen requirements, etc., and heated in a phosphate solution prepared from a mixture of $M/15\ Na_2\ HPO_4$ and $KH_2\ PO_4$ to give a P_H value of approximately 7 is as follows:

4 minutes at 120 C. (248 F.)
10 minutes at 115 C. (239 F.)
33 minutes at 110 C. (230 F.)
100 minutes at 105 C. (221 F.)
330 minutes at 100 C. (212 F.)

The data of similar tests secured with suspensions containing billions of spores produced by the most resistant strains have been used in the compilation

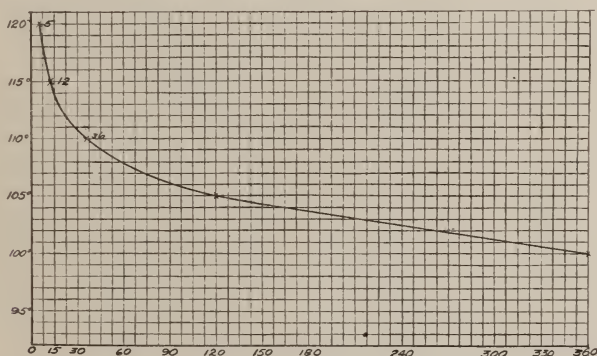


Chart 2.—Ideal destruction curve of *B. botulinus* strains 19, 23, and 97 grown in double strength veal peptic digest gelatine at 37 C. for 10 days, 60 billion in $M/15\ PO_4$ solution, $P_H 7.0$.

of the "ideal destruction curve" shown in chart 2. This curve includes the maximum resistance observed on 1,804 spore suspensions and shows the negative results obtained at the temperatures stated.

VARIATIONS IN THE HEAT RESISTANCE OF SPORES

The heat resistance of the spores of *B. botulinus* varies considerably, depending on several factors, some of which are unknown. The influence of the composition and the reaction of the broths used for the productions of the spores have been studied on 24 different mediums. The following preparations have been tested; pea-juice-peptic digest broth, pea-juice gelatine (10%), pea-juice-peptic-digest gelatine (10, 15 and 20%), pea juice, spinach juice, spinach juice with 2% Berna peptone, spinach-juice-peptic digest, spinach-juice-peptic-digest gelatine (10%), double strength veal infusion with and

⁵ Untersuchungen über pathogene Anaeroben, 1908.

⁶ Centralbl. f. Bakteriöl., O., I, 1920, 84, p. 71.

without peptic digest and gelatine, peptic-digest broth with and without Bernal peptone (1%), diluted pea juice, glucose-peptic digest with and without gelatine, brain natural reaction and brain with 2% peptone, asparagus juice with 1% Difco-peptone, alkaline egg medium, casein digest, beef-heart-peptic-digest broth and milk agar in anaerobic plates (surface growth). In a number of mediums the influence of the P_H , the salt and phosphate content has been investigated. It has been established that the production of abundant spores with a fairly consistent heat resistance is regular in a double strength veal infusion-peptic-digest (equal parts) 10% gelatine with a P_H of 7.4.

The spores which develop in certain mediums are always of very low resistance. Furthermore, it has been repeatedly noted that the spores generated in different flasks of the same medium, inoculated with equal amounts of the same stock culture and incubated for the same period, may show striking differences in heat resistance. These differences appear to be independent of the final reaction of the culture fluid within the range of P_H 6 to 8, the number and microchemical structure of the spores. Strains which sporulate poorly in liquid behave likewise in solid mediums.

TABLE 2
RESULTS OF EXPERIMENTS ON HEAT RESISTANCE OF SPORES

Flask	Final P_H	Millions of Spores Obtained and Heated	Resistance at 105 C. in Phosphate Mixture P_H 7.0		Retarded Germination
			Minutes +	Minutes -	
1	8.31	600	12	18	16 days
2	7.69	244	24	28	14 days
3	8.19	2,324	12	18	16 days
4	8.22	268	24	28	30 days
5	8.21	880	18	24	18 days
6	8.20	600	24	28	24 days
7	8.05	1,100	18	24	24 days
8	1,040	18	24	21 days

+ = survival time; - = destruction time.

One experiment is cited to illustrate this statement. Eight flasks of pea-peptic digest medium P_H 7.4 of the same lot were inoculated with strain 90 and each received 0.1 cc of the stock beef heart culture. The containers were incubated at 35 C for 10 days and the spores of each culture were heated separately in phosphate solutions of the same P_H prepared from the same standard mixture. The results are shown in table 2.

Supplementary tests have furthermore shown that striking variations are obtained when the spore suspensions are prepared and heated in the culture fluid in which they have been produced. For comparative tests it has, therefore, been found advisable to concentrate the spore material of several flasks containing from 800 to 1,500 cc of culture fluid by centrifugalization and to resuspend the sediment in a phosphate buffer solution of a neutral reaction. This phosphate mixture is easily duplicated, undergoes little or no change during the heating, and the spores tested in this solution have, as a rule, possessed the maximum resistance.

Several factors responsible for the peculiar variations in resistance deserve a more detailed discussion than is possible in this summary. They will be presented in the final report.

THE RESISTANCE OF *B. BOTULINUS* IN NATURAL AND ARTIFICIALLY INFECTED SOILS

For practical purposes it appeared advisable to determine the heat resistance of the spores of *B. botulinus* as found in nature. Suitable soil specimens selected from the collection of field samples which have been studied in connection with the problem on the distribution of this anaerobe in the United States have been used for this purpose. Definite amounts of 56 soil specimens originally collected in regions in which human or animal botulism had occurred and known to contain spores of *B. botulinus* have been weighed into large hard glass tubes containing 0.5% glucose-peptic digest-beefheart broth. These tubes have been sealed in an oxygen blast and heated in a bath of boiling water (99.5-100 C.) from 1 to 6 hours. The heated cultures have been incubated at 35 C. and observed daily. Growth has been judged by gas production, turbidity of the medium and the appearance of the meat. The presence of *B. botulinus* has been determined by toxin tests on mice and by deep agar shake cultures.

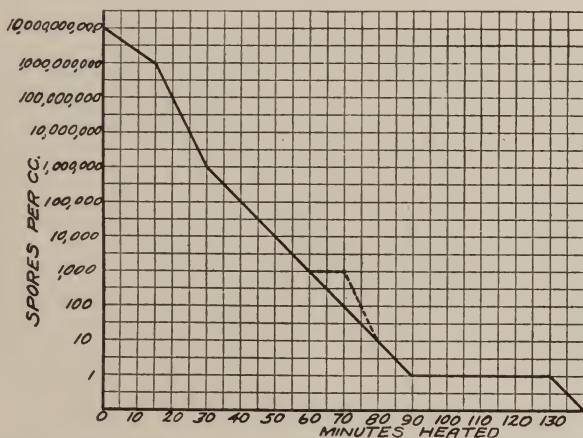


Chart 3.—Death rate of *B. botulinus* spores in phosphate M/15 KH_2PO_4 and Na_2HPO_4 at 100 C.

Three specimens of soil obtained from Colorado, New York and Washington contained a sufficient number of viable spores of *B. botulinus* to furnish toxic cultures after being heated. Two samples survived boiling for 1 hour and only 1 for 2 hours. All the samples heated for 150 minutes at 212 F. proved nontoxic and free from *B. botulinus*. These tests indicate that the average resistance of the spore of *B. botulinus* as found in approximately 20 gm. of naturally contaminated soil is less than 3 hours at 100 C. (212 F.).

Additional tests have been performed on 15 soil specimens, 6 sterile and 9 nonsterile, artificially inoculated with detoxified spores of *B. botulinus* and incubated for from 20 to 30 days aerobically or anaerobically at 27 C. It is known that *B. botulinus* developed in these soil cultures. Samples which contained sufficient toxin to kill guinea-pigs in from 20 hours to 5 days when fed, have been chosen for the heat resistance tests. Equal amounts of the soil suspension cultures have been treated in the manner stated. The data show that 8 of the 9 nonsterile soil samples developed toxic cultures after exposure to boiling water for 2 hours. All tubes heated for 150 minutes have been negative for *B. botulinus*. One sample of the 6 originally sterilized soil

suspensions has become toxic after heating for 1 hour. The tubes heated for 2 hours have remained sterile. The spores of *B. botulinus* produced in sterile or nonsterile soil suspensions were destroyed in less than 3 hours. The second series of experiments fully confirm the results secured on naturally contaminated soil samples.

THE DEATH RATE OF *B. BOTULINUS* SPORES AT 100 C.

Chart 3 illustrates the death rate of the spores of *B. botulinus* suspended in a phosphate mixture and subjected to boiling water (99.5-100 C.). The survival of the spores has been determined in liquid mediums. Irrespective of the fact that the method furnishes only approximate data, it is evident that the mortality of the spores is a gradual process and follows in a general way the laws of logarithmic decline. The majority of spores are not exceptionally heat resistant. A relatively small number in this suspension survives the temperature of 100 C. for more than 90 minutes. Germination of these resistant spores may be

TABLE 3
RESULTS OF TIME-TEMPERATURE EXPERIMENTS

Strain	Grown in the Following Medium	Million Spores per 1 C.c.	pH*	Heated in	Time in Minutes at									
					120 C.		115 C.		110 C.		105 C.		100 C.	
					+	-	+	-	+	-	+	-	+	-
62	Pea-peptic-digest broth	1,300	7.78	Pea-peptic digest 7.78*	1	2	3	4	6	7	33	34	120	130
90	Pea-peptic-digest broth	1,800	8.01	Pea-peptic digest 8.01*	1	2	3	4	9	10	44	48	160	170
47	Pea-peptic-digest broth	1,600	7.60	PO ₄ -6.85	1	2	6	7	15	16	65	70	285	300
97	Double strength veal infusion-peptic-digest gelatine	19,200	7.56	PO ₄ -6.88	4	5	9	10	28	30	90	100	315	330

* Final reaction of medium at the end of growth (10 days at 35 C.).

+ = survival time; - = destruction time.

delayed for several weeks or months. Uneven distribution of the resistant spores may favor the occurrence of so-called "skip-stops" (see paragraph The Influence of Concentration of Spores).

THE TIME-TEMPERATURE RELATIONSHIP

The time necessary to destroy a known suspension of spores in a medium of known hydrogen-ion concentration decreases as the temperature increases. This fact has been established on 7 different spore suspensions. The spores employed in the experiments have been grown either in pea-peptic-digest broth, or in double strength veal infusion-peptic digest gelatine; the suspensions have been heated in the same medium in which the spores have been produced or in phosphate solutions. The results of 4 series are shown in table 3 and chart 4. The curves in chart 4 show the destruction times at the different temperatures.

THE INFLUENCE OF AGE OF THE SPORES ON THE THERMAL DEATH RATE

Four series of experiments have been made to determine the influence of age on the heat resistance of spores held at 35 C., 28 C. and in the icebox (average, 5 C.). In series 1, spore suspensions produced in pea juice-peptic

digest broth have been tested after 4, 8, 12, 16, 20 and 30 days' incubation at 35 and 28 C. The spores of a culture kept at 35 C. for 30 days are less resistant than those incubated for only 4 days. This difference is, however, less marked for spores kept at 28 C. Spore production is slower at 28 C. The maximum resistance is reached on the 8th day and remains fairly consistent when kept at 28 C. The deterioration of the resistance is not influenced by the number of spores. In this series the average count remained practically constant irrespective of the low resistance of the old spores kept at 35 C.

In series 2, the influence of age has been determined by growing strain 90 in pea-peptic digest broth, with and without gelatine, and in neutral brain medium at 37 C., and 28 C. Resistance tests have been made in from 1½ to 186 days. The maximum resistances have been obtained in 1½ to 3½ days in pea gelatine, 2½ days in pea-peptic digest and 6 to 10 days in brain medium. at 37 C. After these periods deterioration of the resistance is rapid, although the number of spores present in the medium remains fairly constant. At 28 C. the maximum resistance in pea gelatine has been reached in 3½ days, and in

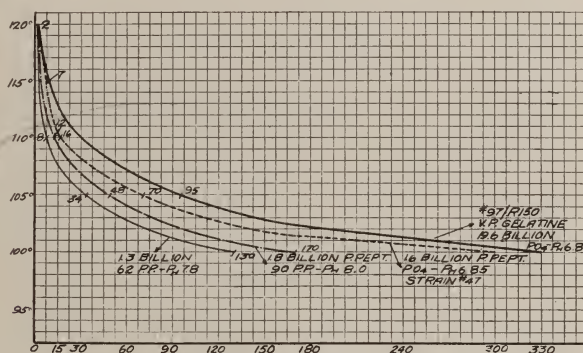


Chart 4.—Destruction curves.

peptic digest broth and brain medium in 8 days. After 15 to 20 days the resistance has dropped rapidly in all cultures irrespective of the number of spores per cubic centimeter of medium.

The third series determined, in duplicate sets, the maximum resistance of spores produced in double strength veal-infusion-peptic digest-10%-gelatine broth at 37 C. Samples have been taken from the same flasks on the 2nd, 4th, 6th and 10th day. Abundant spores have been noted from the 4th day on, and the resistance has remained constant when tested on the 6th and 10th day.

The fourth series confirmed the previous observations. Furthermore, it demonstrated that cultures or spore suspensions kept in the icebox (5 C.) retain their maximum resistance for at least 30 days. Deterioration is, however, noted after a storage of 60 days. Cultures in double strength veal infusion-peptic digest-10%-gelatine broth containing excellent spores exhibit a gradual decrease in resistance after 21 days when kept at 37 C. This deterioration increases on incubation and is quite marked after 60 days. These experiments definitely indicate that the young spores, probably those of the first generation, are the most heat resistant. A well buffered medium, which furnishes abundant food material for progressive growth, produces, as a rule, spores of average or low resistance. Furthermore, any factor, as, for example, body temperature, which

favors after the 10th day the continuous development of new spore-cycles, is conclusive to the production of abundant but poorly resistant spores. Spore suspensions in the moist state are preserved in the icebox for periods not exceeding 30 days.

THE RESISTANCE OF DRIED SPORES

On account of the great variability in the heat resistance of moist spores produced under ideal conditions, it is advisable to employ dried spores for a comparative study of the various factors which are ultimately responsible for their death. Numerous experiments have been conducted to standardize the procedures of dehydration of moist spore suspensions. In brief, it has been found that dried spores are somewhat less resistant than moist spores. Some of the factors responsible for this difference are in the course of investigation. The methods employed to prepare dried spores are briefly as follows: Washed spore suspensions are mixed with sterile sand. The moist material is smeared in Petri dishes and dried in vacuo over H_2SO_4 , $CaCl_2$ or P_2O_5 . Rapid drying

TABLE 4

RESULTS OF TESTS MADE TO DETERMINE RELATIONSHIP OF CONCENTRATION OF SPORES TO HEAT RESISTANCE

Strain	Number of Spores per 1 C c	Resistance in Min. at 105 C.		Resistance in Min. at 100 C.	
		+	—	+	—
90.....	900,000,000	44	48
	9,000,000	34	36
	90,000	18	20
	900	12	14
	9	..	2
90.....	628,000,000	33	36
	6,280,000	33	36
	62,800	24	27
	6,280	15	18
	628	10	15
	63	..	5
97.....	72,000,000,000	230	240
	1,640,000,000	120	125
	32,800,000	105	110
	650,000	80	85
	16,400	45	50
	328	35	40

+ = survival time; — = destruction time.

in from 4 to 6 hours in vacuo at 34 C. (93 F) has produced the best results, and spores with a fairly high degree of heat resistance have been secured. Over a period of 347 days the resistance has remained constant and it appears that perfectly dry spores can be preserved equally well at 37 C., room temperature (20 C.), or in the icebox. Dried spores were found to be excellently suited for comparative studies, and their use can be highly recommended.

THE INFLUENCE OF CONCENTRATION OF SPORES

The relationship of the concentration of the spores to the heat resistance has been determined on 15 different spore suspensions. The results secured in 3 tests are shown in table 4.

From the foregoing data it is evident that the greater the concentration, the greater the resistance. On account of the remarkable variability of the resistance of *B. botulinus* spores produced of one and the same strain under identical conditions, this condition holds true only for the dilutions of a given

suspension. It is a common experience to encounter cultures with less than one million spores, although their resistance surpasses that of others containing several billion organisms per c.c.

In this connection, attention is again called to the phenomenon of retarded germination and the occurrence of so-called "skip-stops" within the first 90 days of incubation. Subcultures prepared from heated suspensions have germinated after an incubation of 378 days at 36-37 C. The cultures produced a virulent toxin and presented typical morphologic and biochemical characteristics.

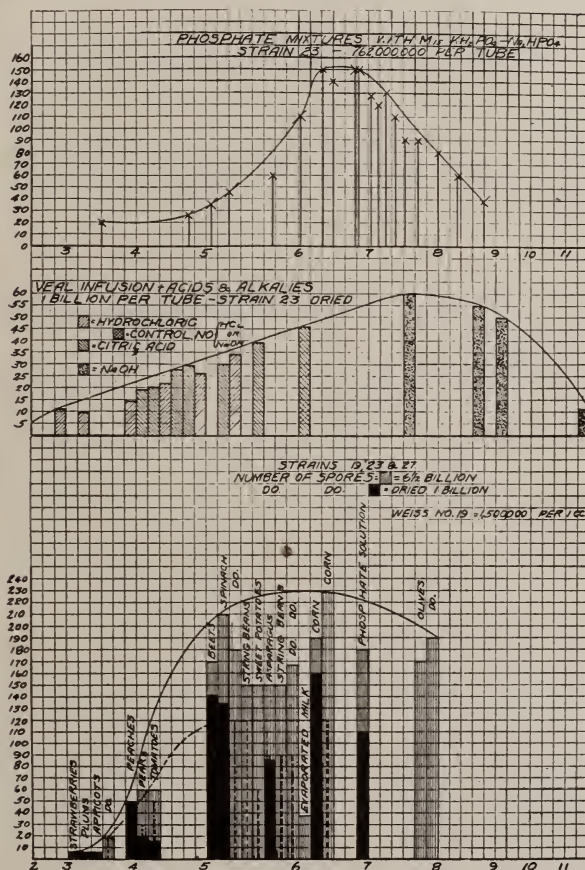


Chart 5.—Resistance of *B. botulinus* spores in phosphate mixtures, veal infusion of varying pH values by the addition of acid or alkali and food juices.

It has been frequently observed that subcultures made from suspensions which have been heated for long periods presented growth sooner than those heated for shorter times. These so-called "skip-stops" are believed to be the result of an uneven distribution of a relatively small number of very heat resistant spores. The occurrence of these "skip-stops" can, in part, be reduced by the preparation of carefully strained, uniform suspensions and by prolonged incubation. Subcultures prepared from diluted unheated or slightly heated spore

suspensions (80 degrees for 1 hour or 100 degrees for 2 minutes) exhibit the phenomenon of retarded germination and "skip-stops." These and similar observations obviously indicate that the germination of a few spores of *B. botulinus* and *B. sporogenes* in suitable culture mediums is irregular and sometimes retarded for considerable periods.

THE EFFECT OF THE P_H ON THE THERMAL DEATH RATE

The effect of the hydrogen and hydroxyl-ion concentration on the heat resistance of spores of *B. botulinus* has been determined by suspending carefully washed spores in 4 different series of solutions of varying P_H values: (a) phosphate mixture series ($M/15 Na_2HPO_4$ and KH_2PO_4) varying from P_H 3.50 to 8.80; (b) 2% Difco peptone solution; (c) double strength veal infusion, and (d) spinach juice (extract of fresh leaves in boiling tap water for 1 hour) with varying amounts of $N/10$ acid or alkali.

Chart 5 illustrates the heat resistance of spores in phosphate mixtures. It is evident that the maximum resistance is on the acid side (about P_H 6.3 to 6.9. Final conclusions cannot be drawn from this series since prolonged incubation may change the available data.

TABLE 5

EFFECT OF HEAT ON SPORES OF *B. BOTULINUS* SUSPENDED IN ACIDIFIED SPINACH JUICE

Hydrochloric Acid				Citric Acid			
P_H	Resistance at 100 C. in Minutes		Retarded Germination, Days	P_H	Resistance at 100 C. in Minutes		Retarded Germination, Days
	+	-			+	-	
Control 5.05	45	50	7	Control 5.26	60	65	10
4.80	45	50	10	4.92	45	50	29
4.50	40	45	8	4.77	40	45	10
4.38	35	40	58	4.69	35	40	9
4.31	24	26	20	4.54	30	35	7
4.16	20	22	8	4.50	30	35	29
4.11	15	20	7	4.37	25	30	8
3.98	10	15	4	4.34	20	25	9
3.81	10	15	5	4.31	20	25	9
3.70	10	15	10	4.31	18	20	9

+ = survival time; - = destruction time.

The effect of 0.003 to 0.05 normal hydrochloric and citric acid and of 0.015 to 0.074 normal sodium hydroxide in double strength veal infusion on the spores is also shown in chart 5. The influence of the hydrogen-ion concentration on the heat resistance is marked below P_H 5.0 and above P_H 10.0. Malic, acetic, succinic and tartaric acid produce in a general way the same effect; below P_H 5.0 the resistance is greatly decreased. Hydrochloric and acetic acids are distinctly more toxic, while succinic, citric, malic and tartaric act in the order named. The behavior of the spores in acid or alkaline peptone solutions is similar to that in veal infusion.

The effect of heat on the spores of *B. botulinus* suspended in acidified spinach juice is shown in table 5. Definite amounts of hydrochloric and citric acids varying from 0.00025 to 0.027 normal were added to the spinach juice obtained as stated to produce a series of P_H values varying from 5.05 to 3.70 for hydrochloric acid and of P_H 5.26 to 4.31 for citric acid. This juice is rich in buffer substances and remained unaltered until 0.003 normal acid was added.

In the P_H range 4.5 to 5.5 little or no effect of the acid is noted; below 4.5 the decrease in the resistance is marked, as shown in table 5. It has been

impossible to test the effect of alkalis on account of the profound chemical changes produced in the spinach juice by the addition of sodium carbonate or hydroxide.

THE EFFECT OF SODIUM CHLORIDE AND POTASSIUM CHLORIDE ON THE THERMAL DEATH RATE

Dried spores of several strains have been exposed to varying concentrations of sodium chloride in double strength veal infusion at different temperatures. No reduction in the heat resistance has been noted until the concentration has reached 8% sodium chloride. Ten and 20% solutions of sodium chloride decrease the resistance, while, on the other hand, the addition of 0.5 and 1% sodium chloride makes the destruction more difficult than in a 2 or 3% solution. These observations are briefly summarized in table 6.

An extensive series of tests has been carried out to determine the effect of holding vegetative forms and spores in 20% sodium and potassium chloride solutions with and without double strength veal infusion and Difco peptone (2%). It is shown that the Na-ion is more toxic than the K-ion, since the resistance is conclusively much less in sodium than in potassium solutions.

TABLE 6
EFFECT OF SALT ON THERMAL DEATH RATE

Spores Suspended in	P _H	Resistance at 100 C. in Minutes	
		+	—
Veal infusion—0.0 NaCl.....	6.03	35	40
Veal infusion—0.5% NaCl.....	5.94	45	50
Veal infusion—1.0% NaCl.....	5.94	50	55
Veal infusion—2.0% NaCl.....	5.83	30	35
Veal infusion—4.0% NaCl.....	5.75	30	35
Veal infusion—5.0% NaCl.....	5.70	35	40
Veal infusion—6.0% NaCl.....	5.65	30	35
Veal infusion—8.0% NaCl.....	5.51	25	30
Veal infusion—10.0% NaCl.....	5.50	20	24
Veal infusion—20.0% NaCl.....	5.06	10	12
M/15 phosphate mixture.....	6.92	105	110

+ = survival time; — = destruction time.

Spore suspensions held in 20% salt solutions become more resistant to heat during an incubation of 60 days at 37 C. Spores of considerable resistance are present even after an incubation of 340 days at this temperature.

Spores heated in peptone solutions without salts or meat extractives exhibit a relatively low heat resistance, as is well illustrated by the data in table 7.

The influence of certain amino groups, polypeptides, on the heat resistance of spores is suggested by these observations and deserves for numerous reasons careful investigation.

THE RESISTANCE OF *B. BOTULINUS* SPORES IN FOOD JUICES

The heat resistance of 4 different spore suspensions of *B. botulinus* has been determined in the following juices of 17 varieties of canned food: corn, spinach, asparagus, string beans, peas, ripe olives, evaporated milk, beets, sweet potato, tomato, sauerkraut, pears, peaches, apricots, plums, strawberries and cherries. The spores have been washed twice in sterile tap water, resuspended in tap water, and 5 c c of the suspension has been added to 45 c c of the food juice.

In chart 5 the results recorded with 2 different spore suspensions are shown in graphic form. It will be noted that above P_H 5.0 some factor other than hydrogen-ion concentration has a profound influence on the time necessary for

the sterilization of foods. In this respect the results differ fundamentally from those previously determined on phosphate mixtures or veal infusion acid and alkali solutions, as shown in chart 5. For example, it is striking that spores heated in ripe olives with a P_H 7.93, corn P_H 6.35 and spinach P_H 5.05 have approximately the same resistance, while, on the other hand, in asparagus with a P_H 5.25 and 5.55 sterilization is more readily accomplished. In food juices with a P_H value below 4.5 the hydrogen-ion concentration has a marked influence on the destruction of *B. botulinus* spores. Careful consideration should be given to these facts in estimating the processing times and temperatures applicable to the practical canning of food.

TABLE 7
HEAT RESISTANCE OF SPORES IN VARIOUS SOLUTIONS

Spores Suspended in	P_H	Resistance at 100 C. in Minutes	
		+	-
Phosphate solution.....	6.92	110	120
Peptic digest broth.....	7.00	120	130
Double strength veal infusion.....	6.70	90	100
2% aminoids.....	5.65	60	65
	5.65	60	65
2% Difco peptone.....	7.40	40	50
	7.08	30	35
2% Witte's peptone.....	7.45	20	25
	7.45	20	25

SUMMARY

The heat resistance of 109 strains of *B. botulinus* (78 type A, 30 type B and one nontoxic) from various sources varies from three to 80 minutes at 105 C. The spores were produced in pea peptic digest broth, P_H 8.0, and heated in a phosphate solution P_H 7.00-7.12.

The heat resistance of 33 strains of *B. sporogenes* varies from 10 to 150 minutes at 100 C.; four to 45 minutes at 105 C. and one to 12 at 110 C.

The heat resistance of 24 strains of *B. tetani* varies from 15 to 90 minutes at 100 C. and three to 25 minutes at 105 C.

Strains of *B. bifermentans*, *B. centrosporogenes*, *B. histolyticus*, *B. oedematiens*, *B. aerofœtidus* and the anaerobe isolated by Bengston are killed within six minutes at 105 C. One strain of *B. welchii* survived 24 minutes at 105 C. but was destroyed in 27 minutes.

The maximum heat resistance of *B. botulinus* spores artificially produced under the most favorable conditions for growth and heated in a phosphate solution of a P_H 7.0 is as follows:

4 minutes at 120 C. (248 F.)
10 minutes at 115 C. (239 F.)
32 minutes at 110 C. (230 F.)
100 minutes at 105 C. (221 F.)
330 minutes at 100 C. (212 F.)

These results represent actual survival times at the given temperatures.

The heat resistance of spores of the same strain of *B. botulinus* varies considerably, depending on several factors, some of which are unknown.

The average resistance of the spores of *B. botulinus*, as found in approximately 20 grams, either of naturally or artificially contaminated soil is less than three hours at 100 C. (212 F.).

The death rate of *B. botulinus* spores is gradual and follows in a general way the laws of logarithmic decline. The majority of the spores are not exceptionally heat resistant.

Young moist spores, probably those of the first generation, appear to be the most heat resistant. Spore suspensions in the moist state are best preserved in the icebox for periods not exceeding 30 days. Prolonged incubation of spores held at 20 C. or 35 C. tends to lessen the resistance.

The heat resistance of dried spores has remained constant over a period of 347 days, and it appears that perfectly dry spores can be preserved equally well at 37 or, 20 C. or in the icebox.

It is evident that the heat resistance increases as the concentration increases determined by dilutions of a given suspension. However, cultures with less than one-million spores may have a greater heat resistance than others containing several billion organisms per c.c.

Subcultures from heated spore suspensions of *B. botulinus* have germinated after an incubation of 378 days at 36-37 C.

The hydrogen ion concentration of different solutions affects the thermal resistance of *B. botulinus* spores.

Spores heated in concentrations of sodium chloride from 0.5% to 6% do not decrease in heat resistance. The presence of 8, 10 and 20% solutions greatly decreases the resistance while 0.5% and 1.0% appear to enhance it. Spores heated in peptone solutions without salts or meat extractives exhibit a relatively low heat resistance.

The heat resistance of *B. botulinus* spores in the juices of 17 varieties of canned food shows a variation from less than 10 minutes to 230 minutes at 100 C.

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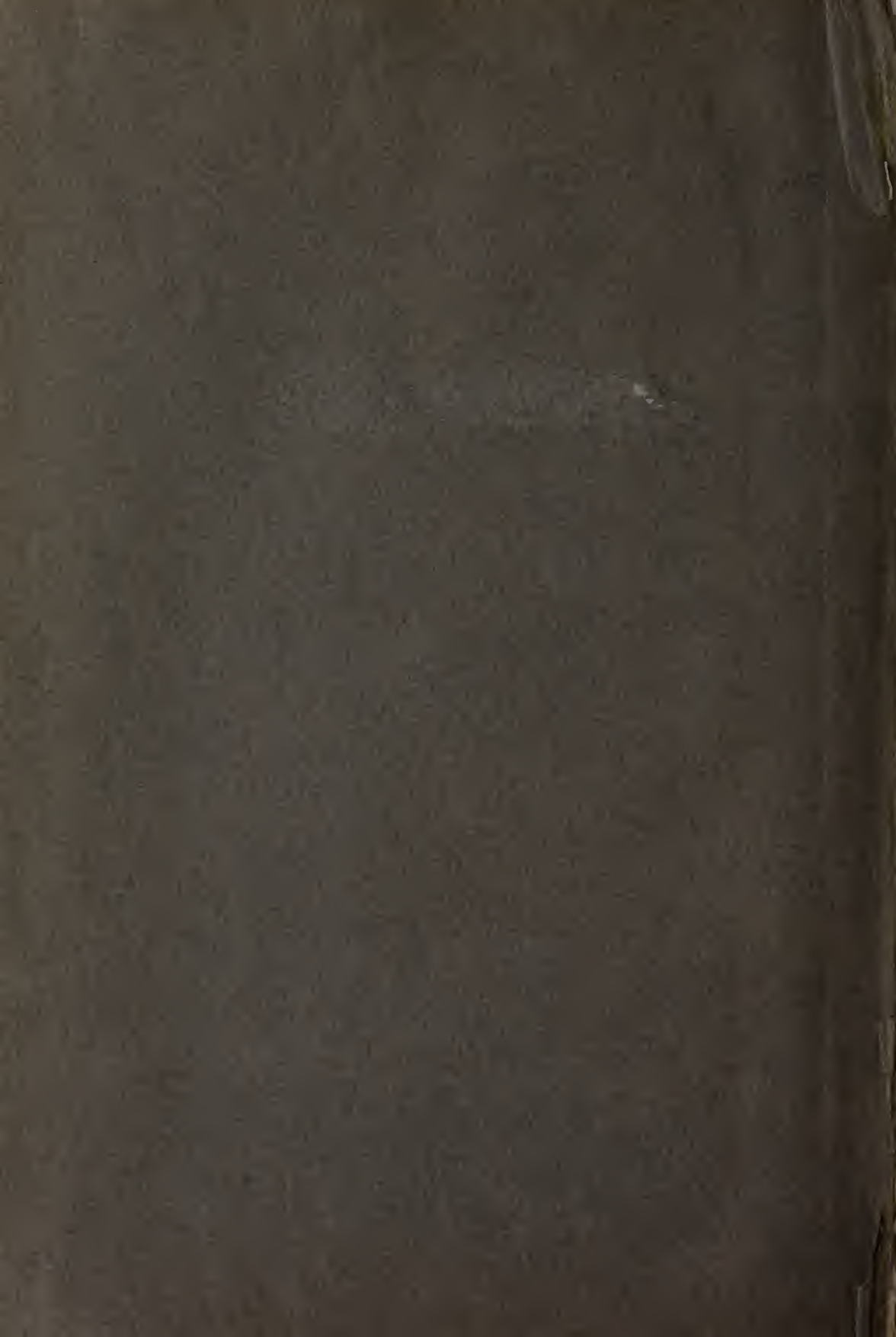
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